Investigating transcription factors in wheat defence against Zymoseptoria tritici fungus.

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Abstract

Zymoseptoria tritici (Septoria) is a devastating fungal pathogen of wheat, causing yield losses of up to 50%. It is classed as a major pathogen threat within the EU. Finding new resistance breeding targets is of upmost importance due to Septoria's ability to evolve resistance quickly.

During this project I used Virus Induced Gene Silencing (VIGS) to study WRKY transcription factors. I identified two that, when silenced, caused a change in Septoria's infection phenotype, TaWRKY19 and TaWRKY9. TaWRKY19 is a resistance factor whereas TaWRKY9 is a susceptibility factor.

To further study the defence network I performed a Yeast 1 Hybrid (Y1H) experiment to identify TFs that bound to TaWRKY19's and TaWRKY9's promoter. Through this screen I found multiple potential binders. I focussed on one, TabZIP2, which bound to TaWRKY19's promoter. Further silencing and infection experiment revealed TabZIP2 to be a susceptibility factor. qRT-PCR experiments were used to study the relationship between TaWRKY19 and TabZIP2, these showed that TabZIP2 appears to be a negative regulator of TaWRKY19 expression.

Results from this thesis offer three potential breeding targets for Septoria resistant wheat, with further TFs also identified for further experimentation.

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Abbreviations

TF – Transcription Factor
PCR – Polymerase Chain Reaction
Y1H – Yeast 1 Hybrid
3AT – 3-amino-1, 2, 3-triazole
VIGS – Virus Induced Gene Silencing
BSMV – Barley Stripe Mosaic Virus

Declaration

This thesis is submitted to the University of Durham in support of my application for the degree of Doctor of Philosophy. It has been composed by myself and has not been submitted in any previous application for any degree. The work (including data generated and data analysis) was carried out by myself except where explicitly stated otherwise.

Statement of copyright

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1. Introduction

1.1 *Triticum aestivum* and Septoria fungus interaction

Triticum Aestivum (bread wheat) is one of the major food sources in many parts of the world and has been cultivated for more than 9,000 years [2]. It is the second most cultivated food crop in the world behind *Orysa sativa* (rice) adding around £2,000million to the UK economy per year (based on sales in 2013)[3]. To meet current demands and future population increases it is predicated that wheat production needs to grow by >1.7% annually, well above the <1% currently being achieved[4].

1.1.1 Wheat

Bread wheat is a hexaploid genome (AABBDD), which originates from the inter species hybridization of *T. urartu* (AA) and a close unknown relative of *Aegilops speltoides* (BB). This tetraploid wheat (*T. turgidum*) then hybridized with *A. tauschii* (DD) giving the hexaploid *T. aestivum* [5,6]. Domesticated wheat has several morphological advantages over its wild relatives. Firstly the grain size has changed dramatically from long and thin to short and fat. These changes are thought to be due to two independent traits – size and shape – which have evolved separately and are under the influence of separate genetic components. Current domesticated wheat has a vastly reduced genetic variation for grain size/shape in comparison to the wild relatives so there is likely to be few further drastic changes[7,8]. The new grains are advantageous as they have increased germination rates and produce larger plants[8]. The second major morphological change relates to the lack of seed shattering in current domesticated wheat. Analysis of ancient wheat remains indicates that it may have taken as long as 2,000 years for this trait to develop, much slower than grain size changes[8,9]. Unsurprisingly lack of seed shattering trait is thought to have evolved after changes to grain size. It is advantageous as harvesting becomes less labour intensive for the farmer[10].

In 2016, world wheat production was predicted to be around 749.5 million tonnes[11] with the average amount of wheat consumed per capita in 2013

(last data point) calculated at 65.43kg[12]. From the cereals, wheat production is second only to maize[11]. Wheat consumption is highest when compared to other cereals. After maize, wheat has the second highest usage for animal feed in 2013, 546 million tonnes of maize were used for animal feed compared to 130 million tonnes of wheat[12]. This shows wheat's importance as a crop, both directly and indirectly on food production. It is also used in alcohol distillation and for non-food applications such as biofuels, thatch roofs and livestock bedding[3].

1.1.2 Zymoseptoria tritici

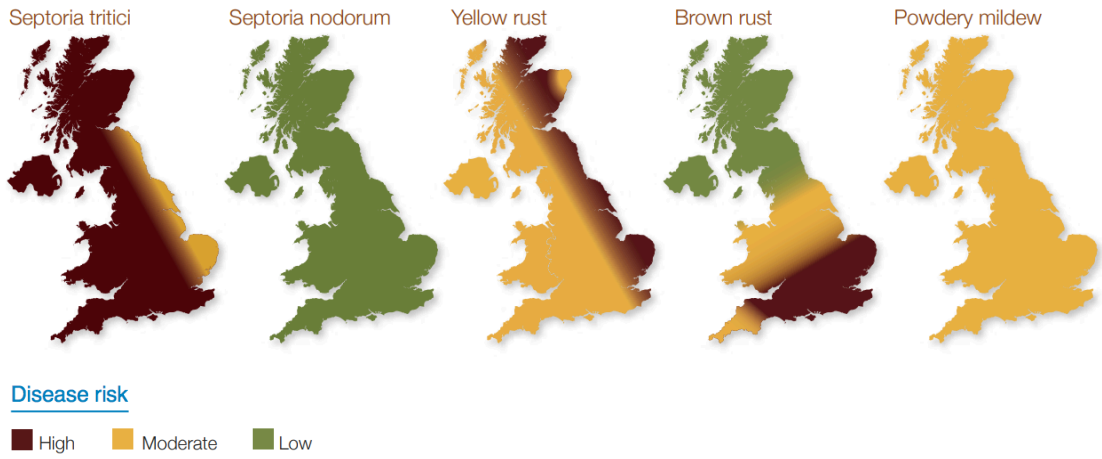

Figure 1.1: Disease severity for wheat pathogens across the UK. Modified from ADHB recommend list 2017/2018[13]

Zymoseptoria tritici is the main threat to UK wheat production (figure 1), with an average potential loss in yield of 50% for untreated crop populations and an average loss in treated crops of 5-10%[3]. In Europe (2014) it is estimated that £1.1billion was spent on fungicides to treat wheat crops, with around 70% of this specifically aimed at preventing Septoria [14,15]. Most of these fungicides were used in just three countries, France, Germany and the UK due to the temperate and humid climate being perfect for Septoria to thrive. Although these multiple fungicide applications come with a great initial cost they lead to a greater reward through enhanced yield (normally reduced by the high disease pressure).. Farmers can expect an average of four times more profit through extra yield, which is a vast increase even

when the cost of the three fungicide applications throughout the growing season is taken into account[15].

Wheat is particularly vulnerable when sown upon straw debris and among early sown susceptible varieties. Septoria has become more devastating since the 1960's with the wider use of commercial, early maturing, semi dwarfing and higher yielding wheat cultivar varieties that are more susceptible to Septoria fungus [16].

Septoria leaf blotch mould is an ascomycete fungus caused by *Mycosphaerella graminicola* (teleomorph stage) in bread wheat in temperate regions. The first visible symptoms of Septoria infection are irregular brown chlorotic spots, which expand as the infection progresses, developing into necrotic lesions on either side of the leaf. Developing with the lesions are the pycnidia, small brown raised fruiting bodies produced on the leaf at the stomata, which release pycnidiospores when mature [16,17]. A sticky medium containing sugars and proteins is produced to protect the spores from drying out. The spores, in the sticky medium, are only exuded from pycnidia on sufficient humidity and appear through an ostiole. Septoria infection relies on water and humidity to progress [16].

The fungus spreads its spores by either wind or within rain splashes [18] infecting the bottom leaves of the wheat plant and potentially going onto infect upper leaves [16]. Over winter or when wheat crops are not present Septoria survives in plant debris as mycelium, in pycnidia and mainly as pseudothecia, which are activated to release the spores upon wetting [14]. It infects in temperate regions with high rainfall, ideally needing moisture for >24 hours and a temperature of 10-20^{0}C [19]. Twenty four to forty eight hours after the spores come into contact with the leaf the Septoria hyphae infiltrate the leaf through the stomata, entering the apoplastic space, where it remains throughout its life cycle. During the early symptomless phase of infection, the first 12-14 days, the Septoria grows as a biotroph using nutrients from the apoplastic space to grow hyphae throughout the mesophyll tissue.

The Septoria genome contains few genes encoding for enzymes that break down the plant cell wall, unlike other fungal pathogens. Instead there is an increase in the protein families for peptidases and alpha amylases[20]. It has been suggested that the fungus relies more on the breakdown of proteins from the apoplast as opposed to carbohydrate degradation to avoid detection in the biotrophic phase[20]. The next stage of the Septoria life cycle is the necrotrophic growth phase where the host's mesophyll cells collapse and die releasing nutrients, leading to leaf surface lesions[14,16,21]. The fungal mycelium rapidly proliferate upon host cell death, with an up regulation in genes encoding for the proteins involved in energy production within the fungal cells occurring at the same time as the release of intracellular nutrients[17]. The cue for Septoria's switch between biotrophic to necrotrophic growth stages is unknown as of yet. The reduction in grain yield is due to the loss of photosynthetically active tissue caused by the fungal infection during necrotrophic growth[22].

Current methods of controlling the infection include the use of chemical fungicides and resistant wheat varieties. Current chemical treatments for Septoria include azoles, SDHIs (Succinate DeHydrogenase Inhibitors) and multi-site fungicides (e.g. chlorothalonil), with a mix of all of these recommended to protect against resistance development[23]. Resistance by some fungi species has already been observed in laboratory and field studies for all different classes. Septoria has already evolved resistance (through single point mutations) against the first two types in either field or laboratory experiments[24]. Quinine outside inhibitors (QoIs) fungicides were widely used until wide spread resistance was reported. Fraaije et al [25] showed that a single point mutation in the *S. tritici* cytochrome *b* gene led to resistance against QoIs quickly, possibly due to QoIs only having one target for their mode of fungicide action. Torriani et al [15] showed at least 4 independent evolutions of the G143A resistant mutation are currently present in Europe. Timing of treatment is also very important as there are few fungicides (e.g. azoles) that can kill Septoria once it has switched into its necrotrophic lifestyle (when the disease becomes visible to the farmers) and

resistance is quickly becoming more prevalent for these. Therefore farmers need to have pre-treated their crops to prevent the initial infection[3].

The *Stb* gene family has been identified in various wheat varieties resistant to several Septoria isolates. So far 21 major STB resistance genes have been identified in wheat, with resistance compatible to different isolates of Septoria. Of these *Stb* genes only one has been cloned, *Stb6*. The gene associated with this resistance locus is TaWAKL4 (Wall-Associated Receptor Kinase Like 4)[26].

Resistance to *Stb* loci can be overcome, for example *Stb4* expressing wheat varieties (which were used for >15 years in California) have been shown to become susceptible to Septoria[27]. It was also shown that *Stb4* resistance was overcome even faster (5 years) in Oregon, therefore resistance depends on both the environment and the Septoria isolates present[28]. A much more important point for breeders regarding resistant varieties is the potential genetic linkage between high yielding genes and susceptibility factors, with current breeding techniques struggling to disconnect this linkage[15]. Yield is the primary target of any breeder, so if a resistance gene has a negative impact on yield it will not be continued in the breeding pipeline. It was also observed that Septoria infection became prevalent at the same time as the major boost in wheat yields during the 1970's[15].

The rapid evolution of Septoria resistance is potentially due to high levels of genome plasticity within the fungus as it undergoes high frequencies of both sexual (ascospore) and asexual (conidia) reproduction[14,17]. Septoria also has the potential to go through 6 growth cycles in a single wheat growth season[3], potentially infecting each new leaf as it emerges. It contains 13 core chromosomes and an extra 8 whose loss appears to have no effect on the fungus so are dispensable to the fungi. Over the course of the growing season around 24% of the final isolates originated from sexual reproduction, which allows gene transfer more readily therefore decreasing the evolution time needed for the population as a whole [29]. Evolution in the 8 dispensable chromosomes occurs more rapidly than in the core chromosomes. They

appear different to the core chromosomes in that although they contain 12% of the genomic DNA they represent only 6% of total genes. They are also made up of around 50% more repetitive DNA and half the density of genes compared to the core chromosomes. There are also major differences between the genes, with the core chromosomal genes being longer, more unique to each other and having different codon usage when compared to those in the dispensable chromosomes. Genes are repeated in both the core and dispensable chromosomes as well although it is unknown which section of the chromosomes these genes originated. Many of the annotated genes from the dispensable chromosomes appear to be transcription factors, functioning in signal transduction or gene regulation. These factors show major differences between the dispensable chromosomes of Septoria when compared to other fungal pathogens[20]. Further studies need to be performed to fully elucidate the function of the dispensable chromosomes.

Wheat varieties have been bred to be resistant to different pathogens but these varieties do not yet have resistance to all the economically important diseases so the use of fungicides is still needed to prevent infection [30]. Rather than breeding in one major resistance gene that could easily be overcome by Septoria the approach of gene stacking multiple minor resistance genes that culminate in a majorly improved resistance is now the focus of some of the major wheat breeding companies[15].

Although many partial and fully Septoria resistant wheat varieties have been bred, their method of resistance is still unknown. Currently there are no model pathogen-plant systems similar to the Septoria-wheat infection, which makes it harder to study [17].

1.2 Plant defence

1.2.1 Background

Plants have several physical barriers that act to defend the plants against pathogens; these are the waxy cuticle and the plant cell wall. Their roles are not limited to defence however, also being involved in water loss reduction, protection against UV radiation and structural support[31,32].

If a pathogen makes it past the physical barriers the plant has other defence responses it can use. Basal resistance to fungal pathogens occurs when pathogen/microbe associated molecular patterns (PAMPs/MAMPs) such as flg22 or chitins are recognised by pattern recognition receptors (PRRs) on the cell surface (FLS2 and CERK1 respectively in Arabidopsis thaliana)[33,34]. This recognition causes a cascade of events that help arm the plant for defence and is known as PAMP/MAMP triggered immunity (PTI/MTI). Once the PRRs perceive a pathogen a cascade of phosphorylation occurs, leading to transcriptional changes. The first step in the cascade is the actvation of a Mitogen-activated protein (MAP) kinase kinase kinase (MEKK or MAP3K). This activation can come directly or indirectly from the PRR. The MEKK then phosphorylates and activates a MAP kinase kinase (MEK or MAP2K). Next the MEKK phosphorylates and activates a MAP kinase (MAPK). Activated MAPK can then go onto phosphorylate other targets such as TFs, leading to global transcription changes (reviewed in[35,36]). The MAPK cascade is not only involved in plant defence, it can also be activated in growth, hormone signalling and abiotic stresses (reviewed in [37,38]). In Arabidopsis there are 60 MEKKs, 10 MEKs and 20 MAPKs[39], it is not a straightforward linear cascade, with different MAPKs etc being involved in different stress activation.

Some pathogens have evolved resistance, via suppression or evasion, leading to successive rounds of evolution by both the plant and pathogen that result in specific plant defences to certain pathogens known as a gene-for-gene relationship [40,41]. Pathogens such as *Cladsporium fulvum* (a pathogen of tomato, *Solanum lycopersicum*) defend themselves by releasing effector proteins such as the tomato (*Solanum lycopersicum*) plant pathogen *Cladsporium fulvum*, which can protect against plant defence by avoiding detection by the plant. *C. fulvum* releases different effectors, such as ExtraCellular Protein 6 (Ecp6), which is a Lysine Motif (LysM) containing protein that binds to the chitin released from the fungal cell walls therefore preventing the plant PRRs from perceiving the pathogen[42,43]. Ecp6 acts to prevent the pathogen from being perceived by the plant, however some effectors attack the plants defence response directly, such as the

Pseudomonas syringae bacterial pathogen effector HopAI1. HopAI1 directly interacts with the MAPKs MPK3 and MPK6. These are essential for transducing the pathogen perception signal leading to many changes such as global transcriptional changes. HopAI1 dephosphorylates MPK3 and MPK6 therefore deactivating the proteins and halting the signal transduction[44].

Although little is known about Septoria's early infection processes some genes have been found to be involved. Wheat homologues of the PRRs Chitin Elicitor Response Kinase 1(CERK1) and Chitin Elicitor Binding Protein (CEBiP) which recognise the PAMP fungal chitin have been identified[45]. Septoria has evolved effector proteins designed to block the receptors from perceiving its presence.

Two proteins (Mg1LysM and Mg3LysM) were first identified based on their similarity to the previously studied LysM effector Ecp6 from *C. fulvum*[46]. Although both are homologous to Ecp6 effector only Mg3LysM Septoria knockout mutants led to impaired and reduced infection in wheat leaves[46]. Moreover when the two wheat receptors (CERK1 and CEBiP homologues) where silenced and infected with Septoria knockouts of Mg3LysM they were able to infect the plant, showing the interaction between the two[45].

Specific resistance to plant pathogens at a species level involves a gene-for-gene interaction[41] where a pathogen avirulence protein (Avr such as Ecp6 and HopAI1) is recognized by a plant resistance protein (R). After this recognition the plant activates a hypersensitive defence response (HR) in which the plant kills off its own tissue surrounding the infection point in an attempt to contain the infection[47]. Susceptible plant species do not have an R gene that corresponds with the pathogens Avr gene and therefore cannot mount an R-gene defence, as it does not recognise the pathogen. This is known as Effector Triggered Immunity (ETI).

Brading et al[48] first studied a potential R gene in the Septoria resistant wheat variety *cv.* Flame, which they designated *Stb6*. It recognised a single gene of the *M. graminicola* isolate IPO323..Stb6 has also been identified as a Wall Associated Receptor Kinase (WAK)-like protein[26]. Wheat varieties expressing

this gene have increased resistance to Septoria isolates expressing the effector *AvrStb6 (*Avirulence*Stb6*) but without the classical hypersensitive response (HR) of killing off the tissue surrounding the infection through programmed cell death (PCD). Instead the wheat leaves show some minor symptoms of leaf chlorosis, but this is not always the case. The defence mechanism that is triggered after the recognition of AvrStb6 is unknown. One potential mechanism is the plant limiting the availability of nutrients, therefore containing Septoria to the apoplastic space and preventing it from forming pycnidia[49]. The *AvrStb6* gene was studied further. It is located in a highly dynamic region in Septoria's genome, being surrounded by many transposable elements[50] It encodes a small cysteine rich secreted protein, consistent with a role as an effector. Whilst a direct interaction between AvrStb6 and Stb6 was not seen in yeast 2 hybrid experiments a link was shown through genetically modifying expression levels. They overexpressed and knockdowned (reduced expression but not a complete knockout) *Stb6* expression in both susceptible and resistant wheat varieties respectively. Infection assays on the transformed wheat lines showed an opposite resistance phenotype to the unmodified wheat, thereby showing Stb6 is a resistance gene [26].

1.2.1 Transcription factors in defence responses

Transcription factors make up an important part of a plant genome. It is predicated that more than 5% of the Arabidopsis genes are TFs[51]. In general plants have a much higher percentage of TF in their genome compared to animals. Of the 19 families of TFs that are shared between plants and animals, 14 families are larger in plants than animals. It should also be noted that this is not due to higher levels of expansion across the entire plant genomes, but more specifically an increase in the percentage of genes represented by TFs. This is not the only group of genes to undergo an expansion; MAPK kinases and defence responsive genes have also increased. This leads to the theory that, due to plants sessile nature, they had to evolve a greater number of genes to regulate stress[52].

The main families, which are defined based on the similarity of their protein sequences and binding preferences[53], of TFs in plants are, in no order, WRKY, NAC, MYB, MADS box, bZIP, AP2/ERF, C2H2 zinc fingers and bHLH. WRKY, NAC, MYB, bZIP, AP2/ERF and bHLH families seem to have a greater focus on immune response regulation compared to other families[54].

TFs have been incredibly important in the domestication of many crop plants. Doebley et al[55] investigated the differences between domesticated crops and their wild relatives, during this they found 6 major genes that are involved in the morphological differences between the crops and their progenitors. Of the 6 genes, five are TFs[55] showing single TF genes can have major effects when modulated.

Once the plants PRRs are activated the cell initiates a rapid defence response. The earliest responses include a Ca^{2+} burst, Reactive Oxygen Species (ROS) production, MAPK cascade and Nitrogen Oxide (NO) production (reviewed in [56,57]). Nearly 50% of the validated targets of MAPK cascade are represented by TFs[58], leading to a big change in the transcriptome post pathogen perception.

TFs are targeted in all aspects of the defence response, starting from direct activation by the receptors. R proteins perceive pathogen effector proteins. Nucleotide-Binding site/Leucine-Rich repeat (NLR) proteins fall into this category of proteins. One such NLR from barley, Mildew Locus A10 (MLA10) can directly interact with two WRKY genes – HvWRKY1 and HvWRKY2. HvWRKY1 and HvWRKY2 act as negative regulators of defence against powdery mildew infection[59]. MLA10 also interacts with another TF, HvMYB6, whose activation leads to an increase in resistance against powdery mildew. Without a pathogen perceived, HvWRKY1 represses HvMYB6. Upon infection MLA10 becomes activated after recognising the Avr protein A10 from powdery mildew fungus, this allows the release of HvMYB8 from HvWRKY1's repression. HvMYB6 can then activate the expression of defence genes, leading to an increase in resistance[60].

The Arabidopsis thaliana TF WRKY33 has been well studied in its role in the early defence response. MPK3 and MPK6 directly phosphorylate WRKY33 upon pathogen detection[61]. This then allows WRKY33 to bind to the promoter of PAD3 (PhytoAlexin Deficient 3)[62] leading to an induction in camalexin biosynthesis, which is also a phytoalexin. Mutation experiments showed that the phosphorylation of WRKY33 by MPK3/6 is needed for full induction of the camalexin biosynthesis gene expression and that this occurs after infection with the necrotrophic fungus *Botrytis cinerea*[61]. Mao et al[61] also showed self-activation by WRKY33, proving the defence response can be rapidly enhanced once it has been activated.

1.2.1.1 WRKY transcription factors

WRKY TFs represent the 7th largest family of TFs in flowering plants[63]. They are defined by having one or more WRKY domains. These highly conserved WRKY domains are 60 amino acids in length. The C terminal of the WRKY domain contains a zinc finger motif and the N terminal has the conserved distinctive heptapeptide WRKYGQK amino acid sequence. There are some variants of this sequence found in plants (such as WRKYGEK and WRKYGKK)[64,65]. TFs with variants of the core WRKY sequences have also been found mostly replacing the middle two amino acids[65-67], however it is speculated that these changes may have an effect on their ability or affinity to bind to DNA[68].

There are 3 different groups of WRKY transcription factors, I, II and III. Group I have two WRKY domains, group II have one WRKY domain. Both group I and II have a zinc finger with the structure C-X_{4-5}-C-X_{22-23}-H-X_1-H. Group III contain 1 WRKY domain however with a different variant of the zinc finger structure domain (C-X_7-C-X_{23}-HX_1-C)[69]. Group II have been further subdivided into IIa-e, differentiated by their phylogenetic relationships in arabidopsis[70].

WRKYs bind to W boxes, (C/T)TGAC(T/C), in the promoters of their target genes. W box elements are prominent in plant genomes. WRKY TFs bind to the GAC core whilst the surrounding bases of the W box determine

recognition for specific WRKY TFs[68]. Some WRKY TFs require a cluster of multiple W boxes before binding, for example barley WRKY38 binds to two adjacent W boxes[71]. Many WRKYs have W boxes in their own promoters and can be cross or autoregulated[61,72,73]. W boxes are also highly present in many defence related genes [70,74]. WRKY TFs do not just bind to W boxes; there have been examples of binding to other promoter elements, such as WT boxes (GGACTTTC)[75,76], Pathogen Responsive Element 4 (PR4, TGCGCTT)[77], SUgar REsponive (SURE) element[78] and WK boxes (TTTTCCAC)[79].

There are only a few examples of WRKYs outside the plant kingdom, with ancient lateral gene transfer predicted as the most likely source[80]. For example, *Rhizopus microspores* (a soil fungus) and some mycorrihizae fungi species (e.g. *R. irregulris*), which both infect plant cells, are some of the non-plant organisms with WRKY genes. Hence why gene transfer is thought to be the source of the unexpected WRKYs[80].

It is predicated that there are 72, 109 and 72 individual (199 including A, B and D homologues) WRKY transcription factors in arabidopsis, rice and wheat respectively[74]. Different WRKYs can act as positive and negative regulator, and sometimes one WRKY can act differently in different pathways. They can also form homo/heterodimers to function in different roles[81]. WRKY TFs do not appear to favour forming heterodimers within their groups, as many examples of heterodimerisation have been experimentally shown across the groups[82]. The formation of different dimers can also allow a switch in the TFs function, in rice OsWRKY62 homodimers are negative regulators of the defence gene DiterPenoid Phytoalexin Factor (DPF), however when it forms a heterodimer with OSWRKY45 it leads to a strong induction of PDF gene expression[81]. This can potentially affect studies in which just one WRKY gene is focussed on, including this thesis, as most of the heterodimers between WRKYs are unknown. Results from studies that change the expression of one WRKY gene may be misinterpreted, as this may mean heterodimers are not formed, leading to a change in the function of an unknown WRKY that would have been part of a heterodimer.

Another example of a WRKY having opposing effects in different pathways is AtWRKY70. It is thought to be involved in defining the balance between the defence hormones, jasmonic acid (JA) and salicylic acid (SA) (to nectrophic pathogens and biting insects or biotrophic pathogens respectively) that generally act antagonistically towards each other (reviewed in [83,84]). Li et al [85] studied AtWRKY70 and its role in defence against the necrotrophic fungal pathogen *Alternaria brassicicola* and the biotrophic fungal pathogen *Erysiphe cichoracearum*. They found overexpression mutants of AtWRKY70 caused increased resistance *to E. cichoracearum* but decreased resistance to *A. brassicicola*. They suggest that AtWRKY70 is involved in the regulation between JA and SA mediated resistance hence the opposing defence results.

AtWRKY70 can also work in partnership with AtWRKY53 and AtWRKY46 to positively regulate defence against the bacterial pathogen *P. syringae*. The functional redundancy of these WRKYs was studied through mutant lines. Double and triple knockout mutants caused enhanced disease susceptibility to *P. syringae* compared to wild type and single knockout mutants[72].

AtWRKY48 however is an example of a negative regulator of basal defence against *P. syringae.* Xing et al[86] used both T-DNA insertion mutants and AtWRKY48 OX mutants to study the effects of AtWRKY48 on *P. syringae* infection growth. Data from this study showed enhanced growth on the everexpressing lines and decreased growth on the knockout mutants. They also examined the expression levels of PR defence related genes, finding again enhanced expression on overexpressing lines and decreased expression on the knockout mutant lines.

WRKY genes have also been studied in crop plants, however less extensively than Arabidopsis. Here are some examples of WRKY genes in crops and their involvement in defence against pathogens.

Rice blast fungus caused by *Magnaporthe oryzae* is a hemibiotroph (similar to Septoria) however *M. oryzae* simultaneously undergoes both biotrophic and nectrophic growth. OsWRKY53 has been studied for its effects on rice defence against rice blast. Overexpression of OsWRKY53 can induce higher

expression of defence related genes and causes enhanced resistance to *M. oryzae* [87].

Another example of a crop WRKY TF is from *Capsicum annuum* (Peppers). CaWRKY1 was discovered to be a negative regulator of defence against the bacterial pathogen *Xanthomonas axonopodis*[88].

Expression levels of 15 out of the 85 identified WRKY TFs of *Brachypodium distachyon* (Purple false broom grass) were upregulated after infection with *Fusarium graminearum* (also a devastating fungal disease of wheat) and *M. grisea*, indicating a role in defence against these fungal pathogens[89].

Within wheat there has been more studies looking at the WRKY TFs involvement in abiotic stress[90-93] however some initial work has been done studying expressional changes under different infections. For instance 4 WRKYs were found to have differential expression post infection with leaf rust (*Puccinia triticina*) fungus[65]. Another study investigated expression changes after SA treatment, finding 9 TaWRKYs whose expression changed at varying time points post treatment[93].

1.3 Techniques for genetic studies in wheat

Studies of wheat are made harder due to its large (16,000Mb) hexaploid genome[94], which is 5 times larger than the human genome and 35 times larger than the rice genome[95]. The hexaploid genome is made up from three genomes designated A, B and D that are all closely related. For wheat genes there are three or n*three copies. This causes a problem with functional redundancy as many of these homologous genes are expressed. Therefore a mutation must be made across each of the three genomes to allow the study of the gene function[96,97].

Reverse genetic techniques are used when the gene sequence is known but the protein function is unknown. Since the rise of sequenced genomes, reverse genetics has become a useful tool in molecular biology. It usually involves modifying the gene or expression of the gene, which can cause a

phenotype change to be studied. Reverse genetic techniques including knock downs act by reducing gene expression (e.g. virus induced gene silencing (VIGS) or RNAi), insertions into the gene sequence (e.g. T-DNA or transposons) or chemical mutagenesis (using ethylmethane sulphonate or ethyl nitrosourea) [98]. Targeting Induced Local Lesions In Genomes (TILLING)[99] lines in bread wheat and durum wheat (cv. Cadenza and cv Kronos respectively) have been developed in a collaboration between John Innes Centre, Earlham Institute, University of California Davis and Rothamsted Research. The plants have been sequenced and are available to buy[100]. However each mutant only has a mutation in one of the chromosomes, therefore extensive backcrossing and crossing with the WT wheat are needed before a gaining a true knockout or knockdown. Transforming wheat involves a more complex and precise transformation process than rice or maize and so it has lagged behind other major crop plants in terms of transformation efficiency[101].

1.3.1 Virus Induced Gene Silencing

VIGS uses the posttranscriptional gene silencing (PTGS) system used by plants to defend themselves against viral pathogens. It allows the silencing of homologous genes[97], which is advantageous in wheat with the 3 genomes. At the nucleotide sequence level, wheat genes can share up to 99% similarity between homologous genes across the three genomes [102]. VIGS can silence genes that have >85% sequence homology, allowing silencing across the three wheat genomes [1,103].

When the virus infects the cell it releases its single stranded RNA into the cytoplasm. The virus then replicates its RNA using a viral encoded, RNA-dependent RNA polymerase, producing sense and anti-sense RNA. The sense and anti-sense RNA may hybridise to form a double stranded viral RNA (dsRNA) molecule, which is recognised by the plant protein DICER (an RNaseIII-type enzyme). DICER then cleaves the dsRNA into small interfering RNA (siRNA), 21-23 nucleotides long [104,105]. These siRNA are then

incorporated into the plant RNAi silencing complex (RISC complex). If the RISC complex comes into contact with any mRNA that are complementary to the siRNA associated with the complex it cleaves these mRNA, leading to mRNA silencing of the viral genes. This slows viral replication and can give the plant immunity to any following infections from this virus [106,107].

VIGS studies can be used to discover the function of a plant protein whose DNA sequence is known by silencing and then studying for any effects [108]. By cloning a small fragment of the selected plant gene into a modified virus vector the plants own viral defence mechanisms can be used to transiently silence endogenous mRNA [109]. Cloning additional fragments into the viral RNA does not affect the virus infectivity [106]. As the modified virus spreads throughout the plant so does the endogenous mRNA, leading to silencing of the gene[109]. Bruun-Rasmussen et al [110] showed that the fragment inserted into the virus was better maintained and more abundant with smaller inserts, particularly if the insert is around 120-500 nucleotides. Fragments smaller than this are less effective silencers and larger fragments are more likely to be lost during viral replication[110,111]. The vector chosen must be from a virus that naturally infects the host plant. Barley Stripe Mosaic Virus (BSMV), a member of the *Hordeiviruses* family[112], naturally infects some monocot species such as barley, wheat, oats and maize [113]. Haupt et al [114] showed that BSMV can spread across barley leaves using GFP expressing BSMV, this indicates that BSMV can be used to silence genes across the leaf.

Strain ND18 of BSMV is currently used for the VIGS[1]. It is comprised of 3 RNA molecules designated α, β and γ. They are each single stranded, positive sense RNAs, which among them encode for 7 major proteins. The viral replicase proteins, that are essential for replication, (αa and γa) are encoded for in the α and γ RNAs. βa, the viral capsid protein, is encoded for on the β RNA, however this protein is not essential for infection in barley.

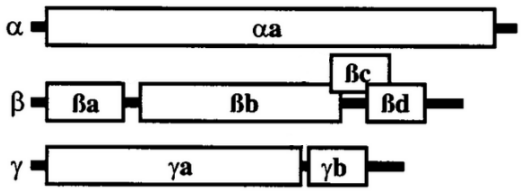

Figure 1.2: A schematic diagram of BSMV αRNA, βRNA and γRNA. Figure modified from Holzberg et al[1].

The Triple Gene Block proteins (TGB) genes, TGB1, TGB2 and TGB3 are all required for viral movement. They are encoded for on the β RNA strand[115,116]. The final major gene in BSMV is located on the γ RNA strand and is named γb. It is a cysteine rich protein involved in pathogenicity, although it is not essential mutations can cause severe changes in infection process in barley and leads to differing expression levels of some of the other major genes of BSMV[115-117].

Holzberg et al [1] were the first to use BSMV to silence genes. The researchers modified the virus to ensure increased silencing efficiency and virulence. To increase silencing efficiency the βa coat protein was deleted. Around 200bp of the gene to be silenced are cloned into the γ RNA downstream of the γb gene in both sense and antisense orientation. To ensure the gene fragment is not translated by the plant post infection a stop codon was inserted in the γb gene. This stops any interference of the γb genes pathogenesis activity. Initial experiments used gene fragments designed against genes that when silenced lead to visible phenotypes, such as Phytoene DeSaturase (PDS) which is needed for carotenoid synthesis for the protection of chlorophylls from photo bleaching[1].

Before silencing wheat with the modified BSMV vector, the BSMV vector is amplified by infection into *N. benthamiana*. It offers a quick, inexpensive and simple way to increase the amount of modified virus for infection into many wheat plants. The insert (of the gene to be silenced) has been shown to be stable in *N. benthamiana* and levels of silencing from the *N. benthamiana* sap were comparable to primary infection of wheat with BSMV [118].

VIGS has successfully been applied using all these changes in many wheat studies investigating pathogens[26,45,111,119-121], insects[122], abiotic stresses[123-125] and yield related genes[126].

VIGS can be more useful than gene knockouts, for example insertional mutagenesis, for a number of reasons. Firstly gene expression silencing does not occur until the plant is infected with the virus, therefore any genes involved in cell growth or development can be studied. It also does not cause

full gene expression silencing as low amounts of residual mRNA are left therefore essential genes may be studied without being lethal to the plant. This can be a negative point as the phenotype seen after silencing may be due to the residual levels of gene expression [97,105]. The viral construct can be generated quickly and the phenotype of PDS silencing through VIGS appears 1-2 weeks post infection. As only a small fragment of the gene to be silenced is cloned into the BSMV vector full knowledge of the target gene is not required[127], which is of high advantage in wheat as the genome has yet to be fully assembled[128,129].

1.4 Aims

The aim of this PhD project is to find potential breeding targets for use in wheat defence against Septoria, with a focus on WRKY transcription factors. Finding multiple breeding targets is desired as gene stacking leads to a decrease in Septoria resistance evolution. The targets will ideally be integrated into the breeding pipeline for KWS and patented for their use in wheat defence against Septoria.

1.5 Objectives

To identify WRKYs potentially involved in wheat defence against Septoria through expression changes..

To successfully silence the identified WRKY genes using VIGS experiments.

Investigate the WRKYs role in defence against Septoria using seedling infection assays.

Start to build up a network of regulation for these WRKY genes through yeast 1 hybrid (Y1H) studies.

Study best candidate TFs identified from the Y1H to elucidate if they have a role in wheat defence through VIGS and Septoria seedling infection assays.

Begin to determine a defence-signalling pathway of TFs based on the Y1H targets and WRKY genes using the silenced plant lines.

2. Methods

2.1 RNA extraction

75mg of frozen wheat leaf tissue was ground to a powder and then 750μl of trizol (Zymo Research, Irvine, USA) was added before vortexing to mix thoroughly. The RNA was extracted using Direct-zol™ RNA miniprep kit (Zymo Research) including the In-column DNase I digestion and eluted in 30μl of DNase/RNase free water. RNA concentration was then measured using Nanodrop ND-1000 spectrophotometer (Labtech, Uckfield, England). The RNA was stored at -80^{O}C.

2.2 cDNA synthesis

1-4ng of RNA was used, with sterile distilled water added to make a final volume of 10μl. To amplify the mRNA 1μl of oligo dT (10mM) (VWR, Radnor, USA) was added to the RNA mixture and then heated at 65^{O}C for 5 minutes before placing on ice. Then 4μl of 5x strand buffer (Invitrogen, Grand Island, USA), 2μl of DTT (Invitrogen), 1μl of dNTP (10mM each) (VWR) and 1μl of RNase OUT (Invitrogen) were added to the RNA mixture. This was then heated to 42^{O}C for 2 minutes before the addition of 1μl of Superscript II (Invitrogen). The mixture was then heated at 42^{O}C for 50 minutes and then 70^{O}C for 15 minutes. The cDNA was then stored at -20^{O}C.

2.3 PCR

All primers were ordered from Eurofins (Huntsville, USA) and used at a concentration of 10pmol/μl. All primers used in this thesis are listed in supplemental table 1.

2.4 Polymerase chain reaction (PCR)

2.4.1 Taq polymerase PCR

Each 20μl PCR reaction comprised of 10μl of reddy mix (Bioline, London, England), 1μl gene specific forward primer (10mM), 1μl gene specific reverse primer (10mM), 1μl of cDNA* (100ug/ul) and 7μl of sterile distilled water, which were spun down before PCR. These were then run on a PCR program (TC-3000G Techne machine), initially at 94^{O}C for 5 minutes. Then 25-35

cycles of 94^{O}C for 30 seconds, X^{O}C for 30 seconds (where X is the annealing temperature, ~3 degrees lower than the primers melting temperature) and 72^{O}C for Y seconds (where Y is calculated from 60 seconds per 1Kb of gene to be amplified) were carried out, followed by 5 minutes at 72^{O}C.

To test PCR primers they were firstly run on a gradient PCR with the annealing temperature set at ~-6 to +2^{O}C degrees of the Tm. The highest temperature that showed a band was selected as the annealing temperature.

The PCR products were analysed and separated on an agarose gel (gel electrophoresis)

For colony PCR a single colony was taken and resuspended in 20μl of sterile distilled water, 1μl of this then replaced the 1μl cDNA used.

2.4.2 Q5 polymerase proof reading PCR

Each 50μl PCR reaction comprised of 10μl of 5X Q5 reaction buffer (NEB, Ipswich, USA), 1μl dNTP's (10mM), 2.5μl gene specific forward primer (10mM), 2.5μl gene specific reverse primer (10mM), 2.5μl of cDNA (100ug/ul), 0.5μl Q5 high-fidelity DNA polymerase (NEB) and 31μl of sterile distilled water, which were then spun down before PCR. These were then run on a PCR program 98^{O}C for 5 minutes. Then 25-35 cycles of 98^{O}C for 30 seconds, X^{O}C for 30 seconds (~3 degrees lower than the primers melting temperature) and 72^{O}C for Y seconds (30 seconds per 1Kb of gene to be amplified). Followed by 5 minutes at 72^{O}C..

The PCR products were analysed and separated on an agarase gel (gel electrophoresis)

2.4.4 Real time PCR

For a 10μl, 50μl of SYBR green (Agilent), 0.5μl forward primer, 0.5μl reverse primer, 0.5μl of cDNA (100ng/μl), 0.15μl ROX (agilent) and 3.35μl of sterile distilled water were added together before being spun down. This was then run on a real time PCR program (StepOne plus Applied Biosystems real time PCR machine) 94^{O}C for 1 minute. Then 40 cycles of 96^{O}C for 6 seconds, 60^{O}C for 10 seconds and 72^{O}C for 10 seconds. Followed by a melting curve step.

The cycle threshold (Ct) values were then normalised to the housekeeping reference gene CDC48[45] and Ef1a.

To test the real time PCR primers serial dilutions of cDNA (x1, x2 and x4) were performed and the Ct values compared.

2.5 Gel electrophoresis

Different percentage agarose gels were made depending on the size of the fragment to be visualized. The gels were between 0.8-1.2%, with the higher concentrations being used for PCR where smaller sized fragments were expected. Per 100ml of 1x Tris-Acetate-EDTA (TAE) buffer (Biorad, West Berkeley, USA) between 0.8-1.2g of agarose (Melford, Ipswich, England) was added and then heated in a microwave until the agarose had dissolved. Per 100ml of solution 0.75μl of ethidium bromide (Fischer Scientific, Waltham, USA) was then added. This was left to set for 30 minutes or until set. The gel tank contained 1x TAE buffer, which filled the tank to above the level of the gel. Five μls of the appropriate hyperladder (either 50bp or 1Kb depending on size of fragment) (Bioline) was pipetted into the first well. Subsequent wells were then filled with 9μl of the PCR reaction*. The gel tank was run at ~100 volts until the dye was over half way through the gel. The fragments were visualized under a UV using a Gene Flash machine and Quantity One program on the computer.

*For PCR reactions using Q5 5μl of 10x loading dye (0.2mg bromophenol blue, 6ml 50% glycerol and 4ml Millipore water) was added before loading into the gel wells.

2.6 Gel extraction

The fragment to be extracted was first run using the method as described in Q5 polymerase proof reading PCR and Gel electrophoresis (2.4.2 and 3.5 respectively). Then the gel was placed on a UV light box and the excess gel removed using a blade. Using the UV light the band was excised and put into a pre weighed 1.5ml tube. The tube was then reweighed to get the weight of the gel. The gel extracted was done following the instructions in the QIAquick

gel extraction kit (Qiagen, Limburg, Netherlands). At the elution stage 30μl of sterile distilled water was used.

2.7 Cloning

2.7.1 Vector ligation

D-TOPO (gateway vector)

To 1μl of D-TOPO (Invitrogen) 5μl of PCR product was added and shook gently. This was then left to stand for 30 minutes at room temperature and put onto ice before transformation.

2.7.2 Miniprep

10ml of LB (Luria Broth) with appropriate antibiotic (Melford) (50μg/ml of kanamycin for D-TOPO, pEarleyGate104 and BSMV) and bacterial colony was grown overnight at 37^{O}C (*E. coli*) or 28^{O}C (*Agrobacterium*). The culture was spun down in the morning for 10 minutes, at 5,000rpm and 4^{O}C and the supernatent discarded leaving the bacterial pellet. The vector was then isolated from the bacteria using QIAprep^{R} spin miniprep kit (Qiagen) eluting in 30μl of DNase free water. The concentration of vector was measured using a Nanodrop ND-1000 spectrophotometer and stored at -20^{O}C.

2.7.3 LR reaction (into gateway donor vector)

To transfer a fragment between a donor vector and a destination vector, 0.5μl of donor vector (50-150ng), 0.5μl of destination vector (150ng) and 1μl of Tris-EDTA (TE) buffer (pH 8.0) were added together. LR clonase II enzyme (Invitrogen) was thawed on ice for 2 minutes and then vortexed for 2 seconds twice. 0.5μls of the enzyme was then added to the vector mixture and vortexed twice briefly before a brief centrifugation. The mixture was then incubated at 25^{O}C for 1 hour. To stop the reaction 0.5μl of Proteinase K solution (Invitrogen) was added, vortexed briefly and then incubated at 37^{O}C for 10 minutes. This was then placed on ice before transformation.

2.7.4 BSMVγ

First the BSMVγ vector was digested with Apa1 (Promega, Madison, USA). 2μl of 10x Promega buffer A, 2μl of acetylated BSA (1/10), 1μg of BSMVγ and up

to 19.5μl were mixed together by pipetting. 0.5μl of Apa1 enzyme (Promega) was then added to the reaction mixture and incubated for 4 hours at 37^{O}C. To inactivate the Apa1 enzyme it was incubated at 65^{O}C for 15 minutes.

Next two reactions were set up at the same time, a vector mixture and a fragment mixture. The vector mixture consisted of 1μl of fragment DNA, 0.5μl of dATP (100mM), 1μl of BSA (1/10 diluted), 1μl of 10x T4 buffer, 2μl of T4 DNA polymerase (1/10 diluted) and 4.5μl of sterile distilled water. The digestion mixture consisted of 4μl of digested BMSVγ mixture, 1μl of dTTP (100mM), 2μl of BSA (1/10 diluted), 2μl of 10x T4 buffer, 2μl of T4 DNA polymerase (1/10 diluted) and 9μl of sterile distilled water. Both the vector mixture and the digestion mixture were incubated at room temperature for 30 minutes before inactivation of the enzyme at 75^{O}C for 15 minutes. 2μl of the vector mixture was then added to the 20μl of digestion mixture before being incubated at 65^{O}C for 2 minutes and then incubated at room temperature for 10 minutes. This mixture was then transformed into chemically competent DH5α *E. coli*.

2.8 Transformation

2.8.1 *E. coli* (DH5α)

200μl of chemically competent cells (DH5α) were thawed on ice (20 minutes) before the addition of 1μl of the vector (either from D-TOPO, LR reaction or BSMV cloning). The cells were then heat shocked at 42^{O}C for 30 seconds before placing on ice. 250μl of S.O.C. medium (Super Optimal broth with Catabolite repression) was added and incubated with shaking at 37^{O}C for 1 hour. The cells were then spread onto LB (lysogeny broth) agar plates containing the appropriate antibiotic for the vector (50μg/ml of kanamycin for D-TOPO, pEarleyGate104 and BSMV) and left overnight to incubate at 37^{O}C.

2.8.2 Agrobacterium

The *Agrobacterium* strain GV3101, pMP90 that is resistant to both rifampicin (25μg/ml) and gentamycin (25μg/ml) was used for all *Agrobacterium* transformations.

200μl of chemically competent cells were thawed on ice (30 minutes) before the addition of 1μg of vector. This was then incubated for 5 minutes on ice, liquid nitrogen for 5 minutes and then 37^{O}C for 5 minutes. To this mixture 1 ml of LB was added before being incubated for 2 hours at 37^{O}C. The cells were then spread out onto LB agar plates containing the appropriate antibiotic for the vector (50 μg/ml of kanamycin for BSMV and pEarleygate104) and left to incubate for 48 hours at 28^{O}C.

2.9 Protein extraction

1g of *N. benthamiana* leaf tissue was collected and frozen on liquid nitrogen. At 4^{O}C the tissue sample was ground to a powder before the addition of 1.6ml of total protein extraction buffer (150mM NaCl (VWR), 1% Igepal CA-630 NP 40 (Sigma Aldrich), 0.5% Sodium deoxycholate (VWR), 0.1% SDS (VWR), 50mM Tris HCl pH 8 (VWR), 1mM EDTA (Sigma Aldrich) and 1 protease inhibitor tablet per 10ml (Roche)) This was ground until homogenous with the thick consistency. A pinch of 1.5w/v of PVPP (PolyVinylPolyPyrrolidone, Sigma Aldrich) was added and mixed in by grinding to inhibit any phenolics in the *N. benthamiana* plant tissue. The mixture was then spun down for 12 minutes at 8,500g, 4^{O}C and the supernatent transferred to a new pre cooled 2ml epindorf tube. The pellet was discarded. Four times SDS page loading buffer (50mM Tris HCl pH 8, 10% glycerol (VWR), 12.5mM EDTA, 2% SDS, 1% β-mercaptoethanol (Sigma Aldrich) and 0.02% bromophenol blue (Sigma Aldrich)) was mixed in by pipetting and then heated for 5 minutes at 98^{O}C before being loaded onto a SDS PAGE gel for protein separation.

2.10 SDS PAGE gel

Gels of 12% were made based on the molecular weight of the proteins (10-70kDa for a 12% gel). For 10ml of 12% gel 4ml H_2O, 3.3ml 30% acrylamide (Sigma Aldrich), 2.5ml Tris.HCl (1.5M pH 6.8), 100μl SDS (10%), 4μl TEMED

(Fischer Scientific) and 100μl of APS (10%, Sigma Aldrich) were added together for the running gel. 7ml of this mixture was pipetted into a 15mm gel mould and 100% isopropanoyl (Fischer Scientific) was syringed on top to create a level gel. This was left for 30 minutes to set before pouring off the isopropanyl and making the stacking gel. For 5ml of stacking gel 3.4ml H_2O, 830μl acrylamide, 630μl Tris.HCl (1M pH 6.8), 50μl SDS (10%), 5μl TEMED and 50μl of APS (10%) were added together. 3ml of the stacking gel mixture was pipetted on top of the running gel and a 15 well comb was placed into the mould. This was allowed to set for 30 minutes. The comb was removed from the gel and the gel placed into the gel tank with 1x running buffer (for 1 litre, 3g Tris Base, 14.4g glycine, 1g SDS and water up to 1 litre after adjusting pH to 8.3) . Up to 30μl of protein was loaded into the wells with the first lane loaded with 5μl of PAGE ruler protein ladder (Thermo Scientific).

The gel tank was run at 60 volts for 3 hours or until the loading dye reached the bottom of the gel. The protein gel was either stained with Coomassie blue or western blotting was used to visualise the proteins bands.

2.11 Comassie staining

The protein gel was stained in Coomassie dye (for 1 litre, 500ml methanol, 100ml glacial acetic acid, 400ml deionised water and 1g Coomassie brilliant blue (Bio-Rad), stirring for 3-4 hours before use) on a horizontal shaker for 30 minutes before destain was added. The gel in destain was left overnight shaking and a photo taken of the gel in the morning.

2.12 Western blotting

The PolyVinylidene DiFluoride (PVDF) membrane (thermo fisher) was activated by soaking and shaking in methanol for 5 minutes. The membrane was then soaked and shook in 1x transfer buffer (for 1 litre, 14.4g glycine, 3g tris base, 800ml deionised water and 100ml methanol added just before use) for 5 minutes before assembly of the transfer. Each of the components for the transfer was first soaked in 1x transfer buffer for 5 minutes. Starting from the black side of the clamp (which faced black side of the electrophoresis holder): sponge, western blotting filter paper (Thermo Scientific), gel,

activated membrane, western blotting filter paper and finally sponge. The gel tank was then filled with 1x transfer buffer and an ice pack placed into the tank along with the clamp containing the gel and membrane. This was run at 30 volts overnight.

The following day the membrane was then blocked with shaking for an hour in 5% milk solution in TBST (for 1 litre, 2.4g Tris base, 8.8g NaCl, 1ml Tween20 and up to 1 litre deionised water after adjusting pH to 7.6) at room temperature. The membrane was washed in TBST to remove any excess milk (1 minute). The membrane was incubated with shaking in the primary antibody (anti YFP with a concentration of 1:10000) for 1-3 hours at room temperature. Three thorough washes of 5 minutes with TBST were then performed at room temperature. The membrane was then incubated with the secondary anti body 1:10,000 (rat for anti YFP) for 1 hour with shaking at room temperature. To wash away any unbound and non-specifically bound antibodies the membrane was washed thoroughly 5 times for 5 minutes in TBST at room temperature. Then 2ml of ECL solution was washed over the membrane for 1 minute. After this the excess ECL solution was removed by dabbing the membrane on tissue and the membrane placed in between transparency film (Nice Day). Photographic film (Fujifilm, Tokyo, Japan) was then exposed to the membrane for differing time depending on the antibody (30 seconds to 5 minutes). The film was then developed to visualize the protein bands.

2.13 Plant growth conditions

The wheat variety Avalon was grown in long day conditions, 16 hours at 24^{O}C and 8 hours at 24^{O}C. Wheat seeds were germinated and grown in John Innes number 2 soil. The *N. benthamiana* plants were grown in long day conditions, 16 hours at 24^{O}C and 8 hours at 24^{O}C. *N. benthamiana* seeds were germinated and grown in John Innes number 2 soil.

2.14 Virus Induced Gene Silencing (VIGS)

To silence wheat the BSMV (Barley Stripe Mosaic Virus) expressing the wheat gene fragment (modified γ BSMV) and transformed in *Agrobacterium*

tumefaciens was first infiltrated into *N. benthamiana* to allow the virus to multiply. The *A. tumefaciens* was grown in an overnight 10ml LB culture and spun down in the morning to get the bacterial pellet. The pellet was resuspended in 10mM $MgCl_2$ to an OD600 of 1.5. Acetosyringone (0.1mM)(Sigma Aldrich) was then added to the mixture, with volume needed being 1/1000 of the total volume of the *A. tumefaciens* mixture and incubated for 2 hours at RT.. An equal amount of α, β and modified γ BSMV were mixed together and then infiltrated into the 4-8th leaf of a 4 week old *N. benthamiana* plant. This was left to grow for a week. The infiltrated leaf was then ground up with water and rubbed onto a wheat leaf (2 weeks old) that had been previously sprinkled with carborundum powder (Fischer Scientific). All the wheat plant leaves were infiltrated and left to grow for 2 weeks to allow the silencing to take effect (successful silencing was seen in the PDS silenced plant).

2.15 Septoria infection

The plants to be infected were trimmed so that only the leaf to be infected was left. The remaining leaves were then stuck down flat onto black card, leaving ~5cm of leaf to be infected. The Septoria strain IPO323 was revived from glycerol stocks onto YPD (1% yeast, 2% peptone, 2% dextrose and 1.5% agar) plates containing no antibiotics and left for 4 days to grow at 18^{O}C. A few streaks of the Septoria were taken from the plate and dissolved into 10ml of distilled water. To measure the concentration of the Septoria spores 10μl of Septoria mixture was pipetted onto a haemocytometer slide. Using an Axkiospop microscope at x20 magnification the spores were counted. The concentration was then adjusted to, on average, 4 spores 0.05*0.05mm square (1,000,000 spores per ml). 0.1% Tween 20 was added to the Septoria mixture.

Silenced wheat plants were prepared by cutting off all leaves except 4-5th. The leaves were then secured onto black cardboard so that a section of roughly 10cm of leaf was available for infection. Using a cotton bud the Septoria was infected onto the leaves by rubbing the mixture onto the leaves (abaxial and adaxial). A new cotton bud was used for each new silenced line

of wheat. The tray under the plants was filled with water and the plants were covered with a lid to generate a high humidity for the Septoria to infect. The lids were removed after 4 days. The plants infected with IPO323 were left for 28 days with pictures taken daily (2pm) and samples taken weekly to check for silencing. The final infected leaves were collected for the spore count, pycnidia count.

2.16 Spore and pycnidia counts

The infected leaves were cut away and suspended in a sealed box containing damp tissue to increase the humidity and cause the fungus to produce spores. This was left at 18^{0}C for 4 days. The pycnidia were counted over a 2cm length of each leaf and averaged for 5 leaves. 5 leaves were then submerged in 10ml of distilled water, vortexed for 2 minutes and left to stand for 3 hours. The leaves were vortexed again for 2 minutes and 10μl of the water loaded onto a haemocytometer slide. The spores were counted for 4 of the 0.2*0.2mm squares on the Akioskop light microscope at x20 magnification and an average taken from these 4 numbers for the spore count.

2.17 Transient expression in *Nicotiana benthamiana*

For transient assay in *N. benthamiana* the gene to be expressed was cloned into a vector containing an YFP N terminal fusion (pEarleyGate104) and transformed into *Agrobacterium*. A 10ml LB culture of this and P19 (RNAi silencing inhibitor) with the appropriate antibiotics (kanamycin, gentamycin and rifamacin) was shaken overnight at 28^{0}C. The culture was then centrifuged for 10 minutes at 4,500rpm, 20^{0}C. The supernatant was then poured away leaving the bacterial pellet. The pellet was washed by resuspending in 10ml of $MgCl_2$ and centrifuged again for 10 minutes at 4,500rpm, 20^{0}C. then resuspended in 10mM $MgCl_2$ to an OD600 of 0.4 and 0.1mM of acetosyrine (0.1M stock in DMSO) was added. The mixture was left for 2 hour at room temperature. A 50:50 mixture of the gene to be expressed and P19 was made and then infiltrated into *N. benthamiana* leaves using a 1ml syringe. The plants were watered and left for 3 days before a small section, 0.5cm^2, was used to visualize the YFP on a SP5 confocal microscope or whole leaves were weighed until 1.5g and collected for protein extraction.

2.18 Confocal microscopy

A section of leaf 0.5cm^2 was mounted onto a slide (Fischer Scientific) in water and a 22x22mm cover slip (Menzel-Glaser, Waltham, USA) was tightly secured over with micropore tape. Either the YFP or GFP setting was used on the LASII software and the x60 OIL objective was selected. A small drop of oil was put onto the objective and the slide was loaded upside down with the coverslip on the side of the objective. Firstly the eyepiece was used to roughly focus the sample using a TLF-GFP light. Then using the software the image was focused fully before capturing it. During the focusing and scanning for an image the speed was set at 700, resolution at 512x512, 1 line average, pinhole airy 1 and bidirectional scanning. To capture a high resolution image the speed was set at 100, resolution at 1024x1024 5 line average and pinhole airy 1.

2.19 Yeast transformation

Two yeast strains (*Saccharomyces cerevisiae*) were used – AH109A[130] and Y187α[132] for transcription factors cloned into the vector pDEST22 (Invitrogen)(prey) and promoters cloned into the vector PTUY1H[133](bait) respectively. The same protocol was used for both transformations.
Fresh (<1 month) yeast colonies (from YPD media plates grown at 30^{O}C) were used to inoculate 10ml overnight cultures of YPD liquid media and grown at 30^{O}C, 200rpm overnight. These were then used to inoculate 50ml YPD liquid media cultures to an OD600 of 0.2-0.3. The cultures were left to grow at 30^{O}C, 200rpm for 4-5 hours or until reaching an OD600 of 0.4-0.6. The cells were then pelleted by centrifugation at RT, 4,000rpm for 20 minutes. The pellet was then washed with 25ml of sterile distilled water and centrifuged. The resulting pellet was then resuspended in 1ml of 100mM sterile LiAC pH7.5 and 100µls was aliquoted into sterile epindorf tubes, 1 per construct. These were centrifuged for at RT for 30 seconds, 11,000g before washing with another 100µl of 100mM sterile LiAC pH7.5. The cells were centrifuged again at RT for 5 minutes at 4,000rpm. The cells were resuspended in 340µl of PEG4000/LiAC solution (240µl 50% sterile PEG 4000, 36µl 1M sterile LiAC pH7.5, 25µl 2mg/ml ssDNA from salmon sperm

(thermo scientific) and 50µl sterile H_20) and 2µl of plasmid (75-150ng/µl). This was then incubated at RT for 25 minutes without shaking before heat shocking at 42^{O}C for a further 25 minutes without shaking. The cells were then pelleted by centrifugation at 42^{O}C, 4,000rpm for 5 minutes. The cells were resuspended in 100µl of 1M sterile sorbitol and spread onto the appropriate selection media plates (-L for PTUY1H vector in Y187α and –W for pDEST22 vector in AH109A). Colonies were expected after 2-4 days of growth at 30^{O}C.

2.20 Yeast mating

A fresh colony (<1 month) from both Y187α (prey yeast) and AH109A (bait yeast) transformed yeast was resuspended together in 0.5ml of YPD liquid media. The cells were vortexed briefly to ensure they were resuspended and then incubated at 30^{O}C with shaking (200rpm) for 20-24hours. 100µl of each mated culture was then spread onto selection media plates (-L-W, -L-W-H, -L-W-H+3AT at 10mM, 20mM, 40mM, 60mM, 80mM and 100mM) and allowed to grow for 2-4 days to see positive interactions.

2.21 Yeast 1 Hybrid

Yeast library (NASC) containing 1,500 *Arabidopsis thaliana* transcription factors (prey) was revived from glycerol stocks using a 96-pin replicator onto –W minimal base media, simultaneously the wheat promoters in PTUY1H, Y187α (bait) were grown on –L minimal media selection media. Both were left to grow for 3 days at 30^{O}C. 96 well plates containing 100µl of YPAD liquid media were then inoculated with the transcription factors whilst the bait was inoculated into 200ml YPAD liquid media. Both were incubated at 30^{O}C for 24 hours shaking at 200rpm. The mating was then performed by using 100µl of the bait culture to inoculate each of the wells of the prey culture and left to incubate at 30^{O}C for 48 hours. The mated cultures were then used to inoculate new 96well plates containing 200µl of –L-W liquid selection media. These were incubated for 24 hours at 30^{O}C. The cultures were then plated onto plates containing selection media to test for mating and interactions (-L-W, -L-W-H, -L-W-H+3AT at 10mM, 20mM, 40mM, 60mM, 80mM and 100mM) and allowed to grow for 2-4 days.

3. TaWRKY19 is a resistance factor against Septoria

3.1 Introduction

Transcription factors (TFs) offer useful breeding tools. As previously mentioned many of the largest advancements in crop domestication came from modulations of TFs. One such example from wheat is the Q allele, which was identified as an AP2 TF[134]. The gene appears to have undergone a minor mutation (one amino acid) between cultivated and wild wheat. It is also more highly expressed in domesticated wheat. The effect of these changes has led to domesticated wheat having shorter stockier spikes that are less likely to shatter. It is also known to affect other growth features such as plant height and spike emergence timing. These changes ensure that farmers can easily mass produce grain and allow mechanical harvesting[135].

WRKY TFs are one of the larger gene families within plants and have been implicated within defence in many different crops, including wheat. TaWRKY49 and TaWRKY62 have opposing roles in defence against *Puccinia striifomris f. sp. tritici* (*Pst*), the fungal pathogen that causes wheat stripe rust. TaWRKY49 silencing lead to enhanced resistance and increased expression of defence related genes whereas the opposite was true for TaWRKY62 silenced wheat[136].

To my knowledge there are no examples of WRKY TFs having a role in defence against *Zymoseptoria tritici*.

VIGS has previously been used within our lab to silence genes that were found to be involved in wheat defence against Septoria [119,137]. The technique is useful due to its speed and ease when compared to the generation of transgenic wheat. It allows silencing across the three homologous genomes simultaneously when the correct silencing fragment is designed.

3.2 WRKY transcription factors and candidate identification

A link between WRKY TFs and Septoria was first identified through a microarray screen performed at Newcastle University data not provided[138]. In this experiment healthy and Septoria infected wheat samples were compared to identify genes and gene families whose expression changed. Along with BZIP and BHLH TFs, WRKYs showed a large change in transcription, both down and upregulated, after Septoria infection (data not shown). WRKY TFs have been studied previously in other plant species and linked to defence. This led as the basis for the project to further investigate individual WRKY genes that could, in turn, be used as future breeding targets for resistant wheat varieties. Septoria is a major threat to wheat production in the U.K. therefore is a major target for seed production companies and their breeders.

Although the wheat genome has been fully sequenced, a final sequence assembly has yet to be produced. Therefore not all genes have been identified within the wheat genome. After an extensive database screen of the most up-to-date assembly of the wheat genome, 72 individual WRKY genes were identified[139], including homologues across the 3 genomes this number goes up to 199. For comparison Arabidopsis has 72 WRKY genes and rice has 109.

WRKYs are arranged into 3 main groups based on the number of WRKY domains and their zinc finger domain structure. These groups are defined as I, II and III, with group II being further subdivided into 5 groups (IIa-IIe) based on Arabidopsis WRKY's phylogeny[70]. A phylogenetic tree comparing the protein sequences of the TaWRKYs was prepared using MEGA7 software. Only one homologue from the three chromosomes was used due to the sheer number and time it would take for the software to align them all. The A genome homologue was chosen if available, although not all genes have homologues across the 3 genomes hence why B and D genome sequences were also included.

Firstly the protein sequences were aligned using MUSCLE software, performed on the MEGA7 platform[140]. Then, after using a prediction tool to determine the most suitable conditions to produce the tree, a phylogenetic

tree was produced with the JTT + Gamma model, using partial deletions and 500 bootstraps. Figure 3.1 shows the tree and is annotated with the WRKY groups, this phylogenetic tree in unrooted. This was determined by blast searches and comparison against the Arabidopsis tree and group definition from Eulgem et al[70]. Some of the protein sequences did not fit into a known group, although they do have a close Arabidopsis homologue. The middle section of the tree appeared to be homologous to Group IIc (TaWRKY49, 51 and 48), Group III (TaWRKY64) and Group I (TaWRKY80), so I did not define them into a group. TaWRKY44 also did not fall into a group nearby on the the phylogenetic tree, being most closely related to Arabidopsis Group I WRKYs. Group III is the largest, with 20 members; this is not the case in Arabidopsis with Group I and IIc being the largest (14 members each). Group IIb is much smaller in wheat than Arabidopsis having only 2 wheat members compared to 7 in Arabidopsis.

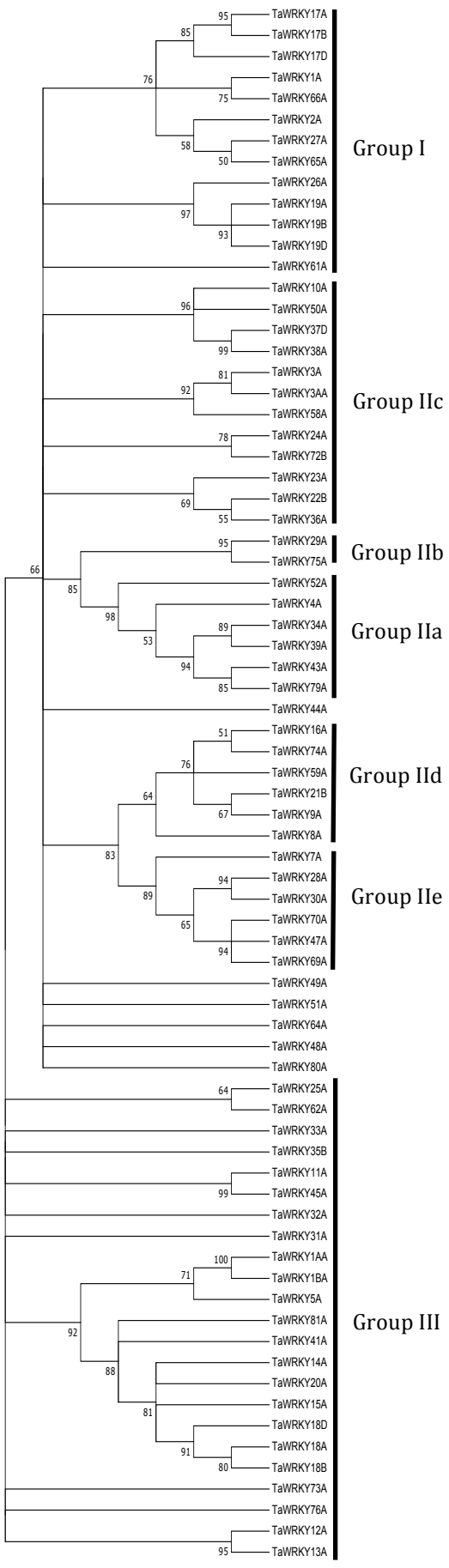

Figure 3.1: Phylogenetic tree of all wheat WRKY proteins. The evolutionary history was inferred by using the Maximum Likelihood method based on the JTT matrix-based model[141]. The tree with the highest log likelihood (-13924.89) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.7246)). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 74 amino acid sequences. All positions with less than 95% site coverage were eliminated. That is, fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 120 positions in the final dataset. Evolutionary analyses were conducted in MEGA7[140]. The tree is unrooted.

Due to the number of TaWRKY genes the first step was to narrow down this number so I could test just those most likely to be involved in wheat defence against Septoria. This was done by searching for previously studied Arabidopsis and rice WRKY genes that are involved in pathogen defence and some involved in abiotic stress, then finding their wheat homologues (table 3.1)[68]. A mixture of abiotic and biotic responsive WRKY genes were chosen as previous work on WRKY genes has shown some are involved in both types of stress, as shown in table 1. OsWRKY53 studies show this WRKY functions in both abiotic and biotic stress conditions.This is potentially beneficial as one WRKY could defend against multiple different stresses through a centralised pathway or could defend against stresses and have a positive role in growth. For instance rice plants overexpressing OsWRKY53 have increased grain size (both length and width)[142] and have increased resistance against rice blast fungus (*Magnaporthe grisea*)[87]. Yield is the main priority for breeding companies, so genes that produce advantages in both grain size and pathogen defence are of particular interest.

As rice is the model crop more WRKY genes from rice, rather than Arabidopsis, were chosen. However some Arabidopsis genes were still investigated as the species has generally been studied in greater depth, therefore having more background and ideas to follow on from than rice.

Wheat gene	Stress	Homologue from Arabidopsis or rice	Expression change in Septoria infected wheat
TaWRKY2	Biotic/Abiotic	OsWRKY53	Upregulated
TaWRKY3	Abiotic	AtWRKY12	No change
TaWRKY9	Biotic	AtWRKY11	Upregulated
TaWRKY11	Biotic	OsWRKY45	No change
TaWRKY16	Abiotic	AtWRKY39	No change
TaWRKY19	Abiotic/Biotic	OsWRKY11	Upregulated
TaWRKY21	Biotic	AtWRKY11	No change
TaWRKY22	Biotic	OsWRKY13	No change
TaWRKY28	Biotic	OsWRKY3	No change
TaWRKY29	Abiotic	AtWRKY6	Upregulated
TaWRKY30	Abiotic	AtWRKY65	No change
TaWRKY31	Biotic	OsWRKY64	No change
TaWRKY36	Biotic	OsWRKY13	No change
TaWRKY43	Biotic	OsWRKY3	No change
TaWRKY79	Biotic	OsWRKY3	No change

Table 3.1: List of TaWRKY genes to be tested and their Arabidopsis or rice homologue. Arabidopsis and rice WRKY genes were selected based on their published roles within either abiotic or biotic stress. The wheat homologues were then identified using blast database searches (KWS).

To investigate whether any of these WRKYs are involved in wheat defence against Septoria the expression profiles of the genes in healthy and infected wheat were studied. Four-week-old wheat seedlings were trimmed so that only the 4^{th} and 5^{th} leaves were left. Half of these plants were then allowed to grow without infection and the other half were infected with $1*10^6$ Septoria spores per ml. The infected plants were placed into high humidity for 3 days to allow the Septoria to germinate and infect through the stomata. Leaf samples were then taken every 2 days from both sets over the next 20 days, with day 0 set on the day of infection. The RNA was extracted and cDNA synthesised from these samples for analysis in semi quantitative and qRT-PCR. A sample period limit of 20 days was chosen as after this time point samples have vastly reduced RNA quality and quantity, leading to poor results. This is due to the leaf tissue dying during the infection process. This experiment was repeated 3 times, resulting in 3 independent cDNA time

courses. All tissue samples were taken between 2-3pm (8 hours into the 16 hour daylight cycle) to ensure the circadian clock machinery was not controlling/influencing any differences in expression.

Primers were designed for each of the 15 identified wheat WRKYs for use in semi quantitative PCR. The healthy and Septoria infected time courses were used to study 15 wheat WRKYs (supplemental figure 1) by semi quantitative PCR. Of these 15, 4 showed a change in expression in infected cDNA compared to healthy cDNA in the initial screen. These were TaWRKY2, TaWRKY9, TaWRKY19 and TaWRKY29. These genes were all seen to be upregulated after Septoria infection around the time of Septoria's switch to necrotrophic growth, which is indicated by the onset of visible symptoms at 12dpi.

After preliminary silencing and Septoria infection experiments were performed on these 4 genes I again narrowed down the targets to just TaWRKY19 and TaWRKY9 (discussed in Chapter 5). I did continue to study TaWRKY2 as the initial experiments showed no differences in Septoria infected when TaWRKY2 was silenced. TaWRKY29 experiments were stopped due to sequence identification errors on my part. These errors were not corrected and TaWRKY29 studies were ceased as I already had two targets and due to time constraints it was decided to stop any further efforts to correct them.

3.3 TaWRKY19

Through preliminary rounds of experiments TaWRKY19 was identified as having a potential role in wheat defence against Septoria. To further investigate this, a more detailed experiment to study the expression changes after Septoria infection using qRT-PCR was performed. This involved using the 3 healthy and infected time courses (used previously in the initial screens).

Firstly, primers for two housekeeping genes for qRT-PCR whose expression did not change after infection with BSMV or Septoria were identified. One set of primers designed against TaEF1a (translation elongation factor 1a) came

from KWS (industrial partner) who have previously thoroughly tested the gene primers under these conditions to ensure their expression does not vary. The other set of primers (TaCDC48, cell division protein 48) was identified from a previously published paper[45]. The primers were used in qRT-PCR on samples that had been treated with BSMV and Septoria[45]. EF1a is part of the EF1 complex, which catalyses the delivery of the correct amino acid carrying tRNA into the ribosome for translation[143]. CDC48 was first identified in yeast as being essential for cell division[144] however it is now known to be involved in many other plant cellular processes[145-149].
Multiple primer pairs for use in qRT-PCR against TaWRKY19 were designed and tested. The first test was to ensure the primers were target specific and only amplified the TaWRKY19 fragment, this was done using semi quantitative PCR and looking for a single band after separation using gel electrophoresis. The second test was for efficiency. This involved using three dilutions of cDNA in a qRT-PCR reaction. As the genes are not highly expressed cDNA dilutions of 1, ½ and ¼ were used of Septoria infected cDNA (10dpi). Primer pairs were selected which showed 90-110% efficiency.
After designing a suitable pair of primers for TaWRKY19, qRT-PCR was performed on the healthy and Septoria infected time courses (Figure 3.2). In the conditions used in these experiments Septoria switches from biotrophic to a necrotrophic growth around 12dpi, indicated by the onset of visible symptoms. In healthy leaf tissue, TaWRKY19 expression levels are low and show no sign of significant change over the 20 days of the time course. In Septoria infected tissue the expression of TaWRKY19 is induced from 6dpi with a peak at 10-12dpi (just before the switch to necrotrophic growth). On average, the highest fold change is 3 times higher compared to the healthy sample (12dpi). The expression is still induced (when compared to the healthy samples) after these time points but not as highly. At 20dpi expression is highly upregulated but this may be due to the cDNA quality obtained from the diseased leaf tissue. From this it can be said that Septoria infection induces TaWRKY19 expression so I can postulate its involvement in wheat defence response against Septoria.

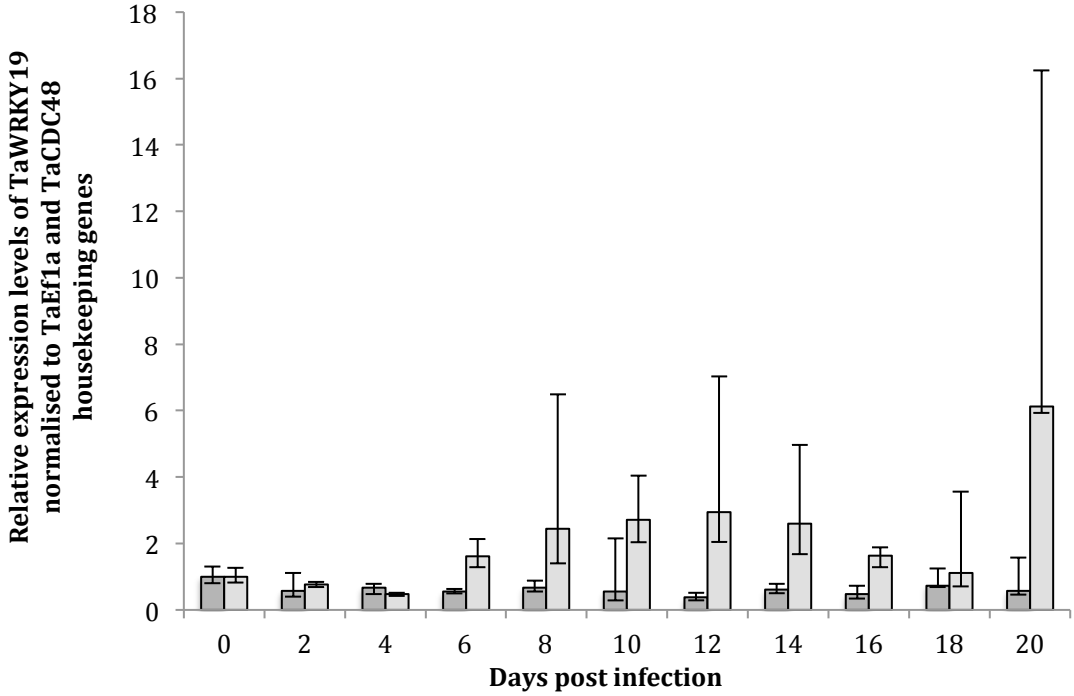

Figure 3.2: TaWRKY19 expression in healthy and Septoria infected time course. RNA samples were collected every 2 days from healthy (dark grey) and Septoria infected (light grey) seedlings of 4-weeks-old. Leaves from three separate plants were collected per sample. qRT-PCR was performed using TaCDC48 and TaEF1a as housekeeping genes. This experiment was repeated 3 times. Two replicate wells for each sample were performed in the qRT-PCR. Error bars represent +/- 1 standard error.

As previously mentioned, WRKY TFs are classified into 3 different groups – I, II and III. The groups are defined based on the number of WRKY domains (one domain for group II and III and two domains for group I) and the zinc finger domain structure (C-X_{4-5}-C-X_{22-23}-H-X_1-H in group I and II and C-X_7-C-X_{23}-HX_1-C in group III) [70,69] that the protein contains. The protein sequence and cartoon structure for TaWRKY19 can be seen in figure 3.3. TaWRKY19 is 468 amino acids long, with a molecular weight of 50.8kDa.
Figure 3.4a shows the protein sequence with the WRKY domains highlighted, The WRKY sequence (red) and zinc domain (blue) have been highlighted separately. TaWRKY19 is a group I WRKY TF as it contains two WRKY

domains (figure 3.4b). The WRKY domains are located towards the C terminal of the protein (figure 3.4b).

A.

MAAGQWSGIGDGGGLWAPPALDSLFPDDQPSPAASALGFFGGSLAQLPSPPPLCG
TALLGYPQDNFDVFHERDLAQLAAQVAQKKELREKQGAGLHHKIGPQLAFSKYSI
LDQVDNSSSFSLATSVLTPQHVSSSVGAALMQGRTLPSHTGSGSVNTGPTGVLQAL
QDSSTTLDSINTGSTGVLEALQGSSITLDRPADDGYNWRKYGQKAVKGGKYPRSY
YKCTLNCPARKNVEHSADRRIIKIIYRGQHCHEPPSKRFKDCGDLLNELNDFDDAK
EPSTKSQLGCQGYYGKPITPNGMMTDVLLPTKEEGDEQLSSLSDIREGDGEIRTVD
GDDGDADANERNAPGQKIIVSTTSDADLLDDGYRWRKYGQKVVRGNPHPRSYYK
CTYQGCDVKKHIERSSEEPHAVITTYEGKHTHDVPESRNRSQATGQHHCKEQTYS
EQSAASFCSSSEKRKYGTAILNDLAF

B.

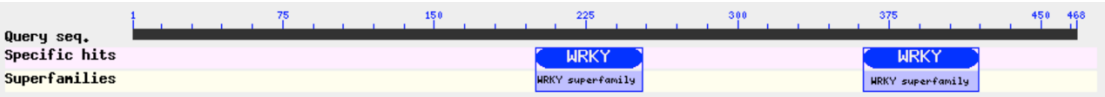

Figure 3.3: Protein sequence of TaWRKY19. A. Protein sequence of TaWRKY19 (468aa). Highlighted in red are the WRKY sequences of the WRKY domain. Highlighted in blue are the zinc finger sequences of the WRKY domains. B. Cartoon representation showing the position of the WRKY domains relative to the protein. This was generated using NCBI protein blast tool[150].

WRKY TFs have high levels of sequence similarity across the family. This can be a problem for VIGS experiments as fragments with >85% homology can silence genes[1,103]. This is due to DICER cleaving the fragments into siRNA of 21-23nt. These fragments are then incorporated into the RISC complex. When the complex comes across mRNA sequences that match the incorporated siRNA it degrades it, ensuring it is not translated. This means there only needs to be stretches of 21-23nt long that are identical between closely related WRKYs for silencing to occur across both. In order to avoid off target silencing of TaWRKY19, the plan was to design the silencing fragments against the 5' and 3' UnTranslated Regions (UTRs)[121]. However, at the time, there was a lack of fully assembled genome unfortunately meaning that the

sequences for the UTRs were unknown for each of the identified WRKYs. To overcome this, Rapid Amplification of cDNA Ends (RACE) experiments were employed. RACE allows the sequences of the UTRs to be identified by adding universal primer binding sites to the ends of the mRNA during cDNA preparation. Using primers against these sites and internal primers at the ends of the known sequences allows amplification of the UTRs using PCR. Once a PCR band has been obtained it can be sent for sequencing (using the internal primer) to give the UTR sequence.

The first step is to decide which RNA sample is most suitable. As can be seen in figure 3.2, TaWRKY19 is upregulated after Septoria infection, just before the switch to necrotrophic growth. Therefore I chose an RNA sample from Septoria infected tissue at 8dpi (just before the switch).

Since the WRKY genes are not highly expressed nested PCR was performed to ensure a high concentration of DNA was available for sequencing. This involved using three different internal gene primers, with each subsequent primer being further towards the start (for 5' UTR RACE) or end (for 3' UTR RACE) of the known sequence and 3 subsequent rounds of PCR. In between each PCR the previous PCR product was diluted 1/10 times in water (deionised) before being used as the sample template for the next PCR. Conditions were kept the same across the 3 PCR experiments.

Figure 3.4 shows the sequence extensions gained from performing the RACE experiments. An additional 192bp of sequence information was identified for the 3' UTR of TaWRKY19 (highlighted in grey). Unfortunately amplicons for the 5' UTRs were not obtained through PCR even with nested PCR.

With this new sequence knowledge, 2 independent non-overlapping silencing fragments were designed for TaWRKY19 (99bp and 83bp), which can be seen in figure 3.4 (highlighted in blue). With two silencing fragments for TaWRKY19 it was hoped that they both show the same phenotype and therefore the assumption is that the phenotype is due to the silencing of the TaWRKY19 gene and not any off target silencing. The silencing fragments were slightly small, however they are still within an acceptable range. From here on in I will refer to these silencing fragments as TaWRKY19A and

TaWRKY19B, this does not have any relation to the genomes of wheat, merely a naming system.

atggcggcggggcagtggtcaggcatcggcgacggcggcggcctctgggccccgcccgcgctcgacagcctc
ttccccgacgaccagccgtcgccggccgcctcggcgctgggcttcttcggtggatccctcgcgcagctcccttcc
cctccgccgctctgcggcaccgcgctcctcgggtacccccaggacaactttgatgtgttccatgaacgagacct
agcacagctggcagcacaagtggctcaaaagaaagagttgcgggaaaaacaaggggcgggattgcatcaca
agattggacctcaactagctttttctaaatacagtatacttgatcaagtggacaactcctcttctttctcattggca
acttcagtgctgacacctcagcatgtcagttcttccgtaggcgcggcattaatgcagggacggactttgccatca
cacactggtagtggtagtgtcaacactggaccaactggagttttacaagcgctccaagattcatccaccactctg
gacagtatcaacactggatcaactggagttctggaagcactccaaggttcatccatcactctggatagacctgc
tgatgatggatacaactggcgtaagtatggacaaaaggcagtcaagggtgggaagtatccaaggagctatta
caaatgtaccctgaattgcccggccaggaaaaatgtagagcactctgcagatagacgaattattaaaataattt
atagaggtcagcactgccatgaaccccccctcaaagaggtttaaagattgtggtgatttattgaatgagttaaatg
atttcgatgatgccaaggagccttcaactaaatcacaattaggttgtcaaggttattatggaaaacctataacgc
caaatggaatgatgacggatgttttattgccaacgaaggaagaggggatgagcaattatctagtttaagtgat
atccgggaaggtgatggtgaaataagaactgttgatggagatgatggtgatgccgatgcaaatgaaaggaat
gcaccaggtcaaaagattatcgtgagtacaacgagcgatgctgatcttttggacgacggctataggtggcgca
agtatggacagaaagtggtgagaggaaatcctcacccaaggagctattacaagtgcacttaccaaggatgcg
acgtcaagaagcatatcgagagatcttccgaggaaccacatgctgtgataactacatacgaagggaagcatac
gcatgacgtgcctgagtctaggaacagaagccaagccacaggtcaacaccactgcaaagagcagacttattc
agaacaatcagctgcaagcttctgcagtagctcggaaaagagaaaatatggaacagccattctgaacgatctc
gccttc**tag**tttggtccccgtgtcttctttaccgaccacggtggtggctcgcgaaagaaagaaagaaacacaat
tcgattggttcttcggtgacgggctgttattgctcatgctctgtttgctgtatattccccactccagtaataactctt
gcatatgcagaaattgtaactgttgaacatgatgggtggtccattgttggaaaaaaaaaaaaaaaaaaaaa
aaaaaa

Figure 3.4: DNA sequence of TaWRKY19. TaWRKY19 CDS sequence is 1,407bp long. RACE PCR was used to identify the 3'UTR of TaWRKY19 (highlighted in grey), adding an extra 192bp sequence. Highlighted in bold are the start and stop codon. Silencing fragments for TaWRKY19 are highlighted in blue.

3.4 TaWRKY19 silencing using VIGS

Once the silencing fragments had been designed it was possible to test them computationally to predict their silencing potential. Although the software is not 100% accurate it provides an indication, before cloning the silencing fragments, as to their TaWRKY19 silencing ability. The software was made available to me by KWS through access to their databases. It calculates each of the potential 22nt fragments (produced by DICER cutting and

incorporated into the RISC complex) possible and ranks them on their silencing potential, with higher number having higher potential silencing ability (0-10 scale). The values for each of the silencing fragments are shown in table 3.2 and 3.3 for TaWRKY19A, TaWRKY19B respectively. The software predicted one siRNA with a high score of 8 and a further six and eight medium strength silencing siRNAs for TaWRKY19A and TaWRKY19B respectively. I therefore continued with both of these silencing fragments.

Start (bp)	End (bp)	Score	GC%
60	82	8	45
33	55	5	35
36	58	5	35
15	37	4	40
17	39	4	40
24	46	4	40
59	81	4	40
35	57	3	35
53	75	3	30
54	76	3	30
32	54	2	35
44	66	1	35

Table 3.2: TaWRKY19A silencing fragment efficiency analysis. Software analysis of the potential 22nt fragments incorporated into the RISC complex ability to silence. Scored 0-10, with 10 representing a high level of silencing efficiency and 1 a low level.

Start (bp)	End (bp)	Score	GC%
62	84	8	45
52	74	6	35
53	75	5	35
56	78	5	35
57	79	5	35
67	89	5	55
48	70	4	30
59	81	4	40
61	83	4	40
49	71	3	30
55	77	3	35
27	49	2	65

Table 3.3: TaWRKY19B silencing fragment efficiency analysis. Software analysis of the potential 22nt fragments incorporated into the RISC complex ability to silence. Scored 0-10, with 10 representing a high level of silencing efficiency and 1 a low level.

To test whether there was a chance of silencing off target genes, I blasted both of the silencing fragments against the most up-to-date version of the wheat genome available (The Earlham Institue[151]) (figure 3.5). Although TaWRKY19A does have some off target sequence homology on other genes they are not as strong as across the 3 genomic versions of TaWRKY19 (A, B and D genomes). The largest stretch of potential off target silencing also does not correspond with any of the predicted stronger silencing siRNAs (table 3.2). TaWRKY19B fragment only has homology with TaWRKY19, again across each of the 3 genomes. This is one aspect in which VIGS is highly useful; even though each of the genomes may have a slightly modified version of TaWRKY19 gene it is still possible to silence each homologue in one experiment due to the flexible nature of VIGS.

A.

TaWRKY19

```
TTGGTCCCCGTGTCTTCTTTACCGACCACGGTGGTGGCTCGCGAAAGAAAGAAAGAAACACAATTCGATTGGTTCTTCGGTGACGGGCTGTTATTGCTC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TTGGTCCCCGTGTCTTCTTTACCGACCACGGTGGTGGCTCGCGAAAGAAAGAAAGAAACACAATTCGATTGGTTCTTCGGTGACGGGCTGTTATTGCTC
```

TaWRKY19

```
TTGGTCCCCGTGTCTTCTTTACCGACCACGGTGGTGGCTCGCGAAAGAAAGAAAGAAACACAATTCGATTGGTTCTTCGGTGACGGGCTGTTATTGCTC
|||||||| ||||||||||||||||||||| |||||||||||||||||||||||||||||||||||||| |||||||||||||||||||||||||  |||||
TTGGTCCCGGTGTCTTCTTTACCGACCACAGTGGTGGCTCGCGAAAGAAAGAAAGAAACACAATTCGGTTGGTTCTTCGGTGACGGGCTGTTGCTGCTC
```

TaWRKY19

```
TTGGTCCCCGTGTCTTCTTTACCGACCACGGTGGTGGCTCGCGAAAGAAAGAAAGAAACACAATTCGATTGGTTCTTCGGTGACGGGCTGTTATTGCTC
|||||||| |||||||||| |||||||| |||||||||||||||||||||||||||||| |||| |||||||||||| |||||||||||||||| ||||||
TTGGTCCCGGTGTCTTCTTCACCGACCATGGTGGTGGCTCGCGAAAGAAAGAAAGAAGCACAGTTCGATTGGTTCATCGGTGACGGGCTGTTGTTGCTC
```

Non coding region

```
AAAGAAACACAATTCGATTGGTTC--TTCGGTGA
|||||||||||||||||||||||||  || |||||
AAAGAAACACAATTCGATTGGTTCAGTTTGGTGA
```

Gene of unknown function

```
CTCGCGAAAGAAAGAAAGAAACACAATTCGATTGGTTCTTC
|||||||||||||||| ||||| ||  ||| || || |||
CTCGCGAAAGAAAGAAAAAAACAGAAAACGAGTGTTTTTTC
```

B..

TaWRKY19

```
TGCTCTGTTTGCTGTATATTCCCCACTCCAGTAATAACTCTTGCATATGCAGAAATTGTAACTGTTGAACATGATGGGTGGTC
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
TGCTCTGTTTGCTGTATATTCCCCACTCCAGTAATAACTCTTGCATATGCAGAAATTGTAACTGTTGAACATGATGGGTGGTC
```

TaWRKY19 chromosome

```
TGCTCTGTTTGCTGTATATTCCCCACTCCAGTAATAACTCTTGCATATGCAGAAATTGTAACTGTTGAACATGATGGGTGGTC
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||| ||||||||| ||||||||
TGCTCTGTTTGCTGTATATTCCCCACTCCAGTAATAACTCTTGCATATGCAGAAGTTGTAACTGCTGAACATGAAGGGTGGTC
```

TaWRKY19

```
TGCTCTGTTTGCTGTATATTCCCCACTCCAGTAATAACTCTTGCATATGCAGAAATTGTAACTGTTGAACATGATGGGTGGTC
|||||||||||||||||||||||||||||||||||||  ||||||||||||||||| ||||||||| ||||||||||||||||||
TGCTCTGTTTGCTGTATATTCCCCACTCCAGTAATATATCTTGCATATGCAGAAGTTGTAACTGCTGAACATGATGGGTGGTC
```

Non coding region

```
TCTTGCA--TATGCAGAAATTGTAACTGTTGA
|||||||  |||||||||||||||||||| |||
TCTTGCAAATATGCAGAAATTGTAACTGATGA
```

Figure 3.5: Blast search of TaWRKY19A and TaWRK19B silencing fragments. The Earlham Institute's wheat genome[151] was used to blast search the DNA sequences of A. TaWRKY19A and B. TaWRKY19B. Parameters were set to identify homology of sequences over 16bps long against the cv. Chinese Spring wheat genome sequence. This is based on the size of siRNA produced by DICER cleavage[104,105].

BSMV is made up of 3 different single stranded, positive sense RNA molecules, designated α, β and γ. Previous studies have modified the virus by deleting the βa coat protein gene to increase silencing efficiency[1]. They also introduced cloning sites so that a gene fragment (from the gene to be silenced) can be introduced downstream of the γb gene. Holzberg et al[1] introduced a stop codon at the end of the γb gene to ensure the plant does not translate the gene fragment after infection and stopping any interference to the γb genes pathogenesis.

Primers were designed to amplify the silencing fragments. The primers contain extensions on the 5' end for use in restriction enzyme cloning. To clone the silencing fragments I used cDNA from 8dpi and Q5 high fidelity DNA polymerase. The PCR was performed following the protocol as seen in chapter 2.4.2 with the following condition for the variable steps, an annealing temperature of 55^0C, an extension time of 20 seconds and 30 cycles. The PCR product was then run on a 1% agarose gel for DNA size separation. Figure 3.6 shows the resulting band from this PCR. The band sizes expected were 99bp and 83bp for TaWRKY19A and TaWRKY19B silencing fragments respectively. The bands were then isolated from the gel and extracted using a gel extraction kit.

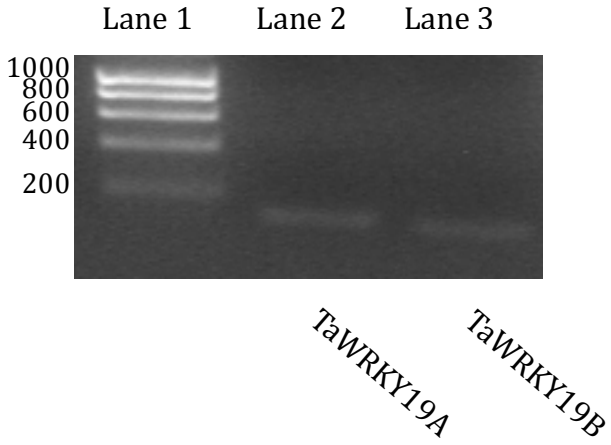

Figure 3.6: Gel of cloning PCR for TaWRKY19A and TaWRKY19B silencing fragments. PCR products were run on a 1% agarose gel in 1xTAE buffer for size separation and visualised using UV light with quantity one software. A 1kb hyperladder was used for size indication (lane 1). The expected size for TaWRKY19A (lane 2) and TaWRKY19B (lane 3) was 99bp and 83bp respectively.

The DNA was then cloned into the modified γ strand of RNA using restriction enzymes before being transformed into DH5α *E. coli* cells. The transformed cells were grown on kanamycin selection plates (the selection marker was also introduced by Holzberg et al [1]) and colonies checked for presence of the γb RNA molecule containing the silencing fragment. This check was performed by PCR, using primers previously designed for sequencing[1]. The primers amplify either side of the inserted fragment, therefore when PCR is performed on the colonies and on the empty BSMV γ vector a shift in the size of the band should be seen for colonies containing the silencing fragment corresponding to the size of the fragment. This can be seen in figure 3.7, with a slight increase in size for colony 5 in BSMV:TaWRKY19A and colonies 3, 4, and 6 in TaWRKY19B when compared to the BSMV empty vector (lane 14).

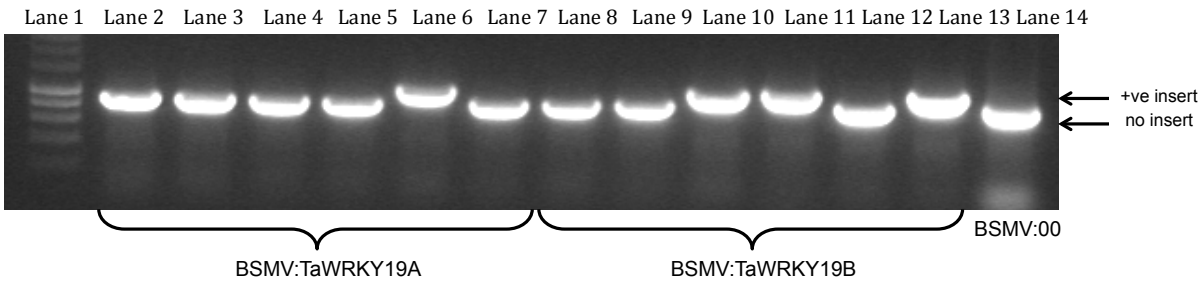

Figure 3.7: Gel of colony PCR for BSMV:TaWRKY19A and BSMV:TaWRKY19B. Primers against either side of the site of insert for the silencing fragment were used in the PCR (BSMV sequencing). PCR products were run on a 0.8% agarose gel in 1xTAE buffer for size separation and visualised using UV light with quantity one software. BSMV:00 was used as a positive control for the PCR (lane 14) and a negative control for the insert. Positive inserts show an increase in size as seen in Lanes 6, 10, 11, 13. A 1kb hyperladder was used to indicate the size of the DNA fragments (lane 1).

I proceeded to grow colony 5 for BSMV:TaWRKY19A and colonies 3 and 4 for BSMV:TaWRKY19B overnight in LB media with kanamycin. I then isolated the vector from the cultures and sequenced the insert, using the BSMV sequencing primers to confirm correct DNA insertion sequence. After the sequence was confirmed, the constructs were transformed into *A. tumefaciens* ready for infiltration into *N. benthamiana* plants.
Now the silencing constructs had been generated they were used to silence wheat WRKY19 before the Septoria infection experiments. Silencing occured within 2 weeks of treatment. To ensure the growth conditions were suitable for silencing a positive control was also performed at the same time on a separate wheat plant. The positive control involved silencing of Phytoene DeSaturase (PDS) gene, which, when silenced, has a distinctive photobleaching phenotype (figure 3.8). The silencing construct for this gene (185bp of PDS gene) was obtained from Rothamsted Research (Dr. Kostya Kanyuka) and has successfully been used previously in our laboratory[119,137].

Even though the growth conditions for silencing were already set up in our growth rooms a PDS silencing control was used to ensure the conditions had not changed unexpectedly and caused a lack of silencing, therefore saving time processing RNA, cDNA synthesis and qRT-PCR.

Figure 3.8: PDS silencing photobleaching phenotype. 2-week-old seedlings were treated with BSMV:PDS and left for two weeks to allow silencing of the PDS gene. Silencing leads to a photobleached phenotype.

Two weeks after the silencing treatment leaf tissue samples were collected for RNA extraction and cDNA synthesis to test the expression levels of the TaWRKY19 in BSMV:00, BSMV:TaWRKY19A and BSMV:TaWRKY19B treated wheat. qRT-PCR was used to measure the gene expression of TaWRKY19, this is shown in figure 3.9. The results are from three independent silencing experiments. TaWRKY19 expression in wheat treated with TaWRKY19A and TaWRKY19B showed a 38% and 45% reduction in expression compared to the BSMV:00. These reductions are statistically significant to 90% and 95%

for TaWRKY19A and TaWRKY19B respectively (p values of 0.094 and 0.011 respectively). TaWRKY19 expression is low without infection; therefore it may be possible that the level of silencing increases when TaWRKY19 is more highly expressed after infection. Silencing relies on the RISC complex coming into contact with the mRNA; hence if there is an increase in TaWRKY19 mRNA then the levels of silencing may increase. Also since I am working on transcription factors a small difference in expression can lead to a large difference in downstream gene expression. Similar levels of silencing have been previously published and led to differences in Septoria infection[45], hence I continued with the experiment and silencing fragments selected.

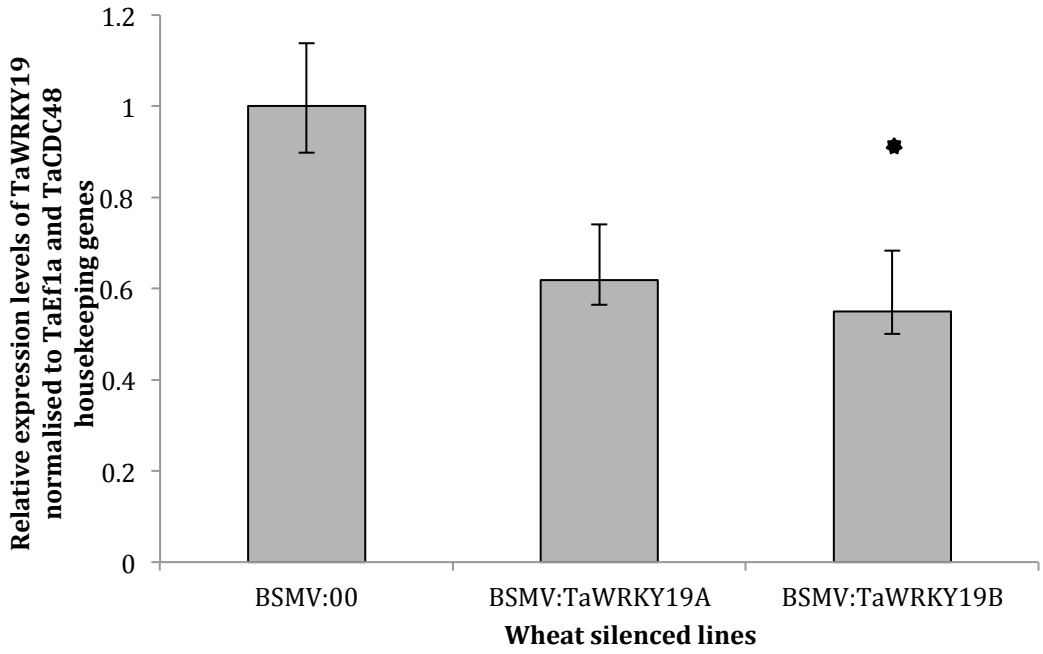

Figure 3.9: qRT-PCR to show silencing of TaWRKY19 in wheat. Shown are the fold changes of TaWRKY19 in wheat silenced with BSMV:TaWRKY19A and BSMV:TaWRKY19B compared to BSMV:00 control. RNA was extracted two weeks post silencing treatment (4-week-old-seedlings). One leaf from three separate plants was collected per sample. qRT-PCR was performed using TaCDC48 and TaEF1a as housekeeping genes. This experiment was repeated 3 times. Two replicate wells for each sample were performed in the qRT-PCR. Error bars represent +/- 1 standard error. Asterisks are used to denote a results difference from the control of $p<0.05$.

3.5 TaWRKY19s role in Septoria infection defence

To test whether TaWRKY19 has a role in defence against Septoria the silenced wheat seedlings (4-weeks-old) were infected with Septoria. The Septoria strain IPO323 was used. Seedlings were then prepared by cutting off all leaves except the 4-5th (based on age, i.e. first leaf to grow = 1st). The wheat seedlings that have been silenced with PDS always show the most severe photobleaching symptoms (figure 3.8) on the 4-5th leaves hence why they were chosen for the infection assays in other silenced wheat lines.

The silenced wheat leaves were then infected with Septoria. The infection process was followed, with photos taken daily (2-3pm) to assess for initial onset of visible symptoms. The onset of symptoms offers one assessment of infection progression. Figure 3.10 shows a representation of the average progression of symptoms in wheat treated with BSMV:00, BSMV:TaWRKY19A and BSMV:TaWRKY19B.

Infection symptoms for TaWRKY19 silenced plants are earlier onset than BSMV:00 treated plants (figure 3.10). Visible symptoms for BSMV:00 appear at 12dpi whereas BSMV:TaWRKY19A and BSMV:TaWRKY19B silenced plants show symptoms after 11 days. The infection follows a similar time scale for both treatments, with the wheat leaves showing full necrosis after 20dpi for BSMV:00 and after 19dpi BSMV:TaWRKY19A and BSMV:TaWRKY19B treated plants. The timescale of infection progression is important as during wheat's growth season the Septoria goes through multiple life cycles, with each subsequent infection occurring on the newly emerging leaves. It is most important, for yield, to protect the flag leaf (final leaf to emerge). Therefore changes in infection speed can have a big effect on overall yield and severity of the infection on the farmer's crop.

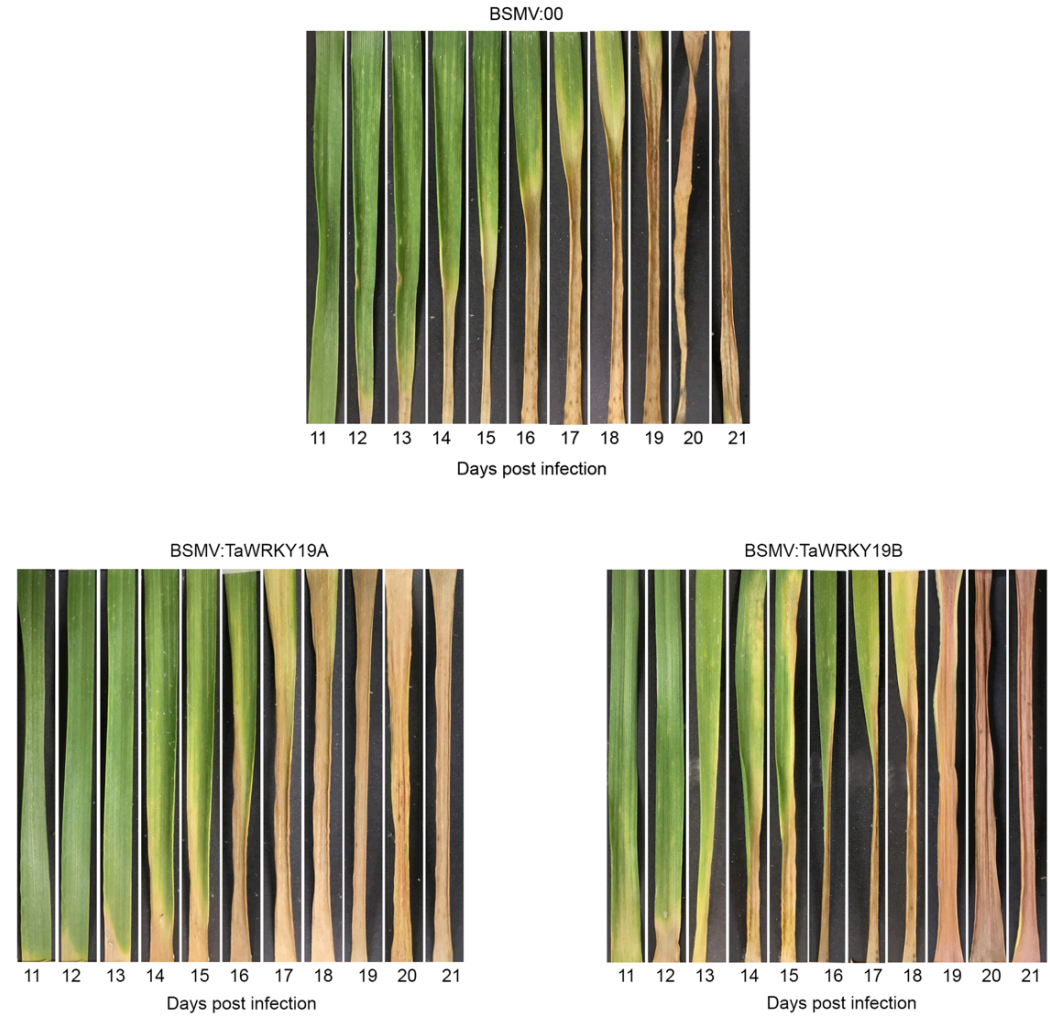

Figure 3.10: Septoria infection symptoms on TaWRKY19 silenced wheat. Four-week-old-seedlings, which had undergone silencing treatment (BSMV:00, BSMV:TaWRKY19A and BSMV:TaWRKY19B) were stuck down onto black card and infected with Septoria (abaxial and adaxial sides of the leaf). The seedlings were grown under high humidity conditions to encourage Septoria infection. The infection was then followed daily, with photos taken between 2-3pm. In this figure the initial stages of the infection symptoms are shown, from 11dpi until 21 dpi. Photos are representative of the symptoms seen in 3 independent experiments.

After the onset of visible symptoms the seedlings were kept in the same conditions until 28dpi when the infected leaf section was cut away. These were then incubated in high humidity (>90%) for a further 4 days to encourage the Septoria's pycnidia to form. The pycnidia were then counted over a 2cm leaf length (figure 3.11). TaWRKY19 silenced leaves have, on average, more pycnidia than BSMV:00 silenced plants with 26, 41, and 37 for BSMV:TaWRKY19A, BSMV:TaWRKY19B and BSMV:00 on average respectively. There is a 55% and 40% increase for BSMV:TaWRKY19A and BSMV:TaWRKY19B respectively when compared to the BSMV:00 control. These numbers are statistically significant to 99% confidence (p values 6.62E-04 and 2.07E-03 for BSMV:TaWRKY19A and BSMV:TaWRKY19B respectively).

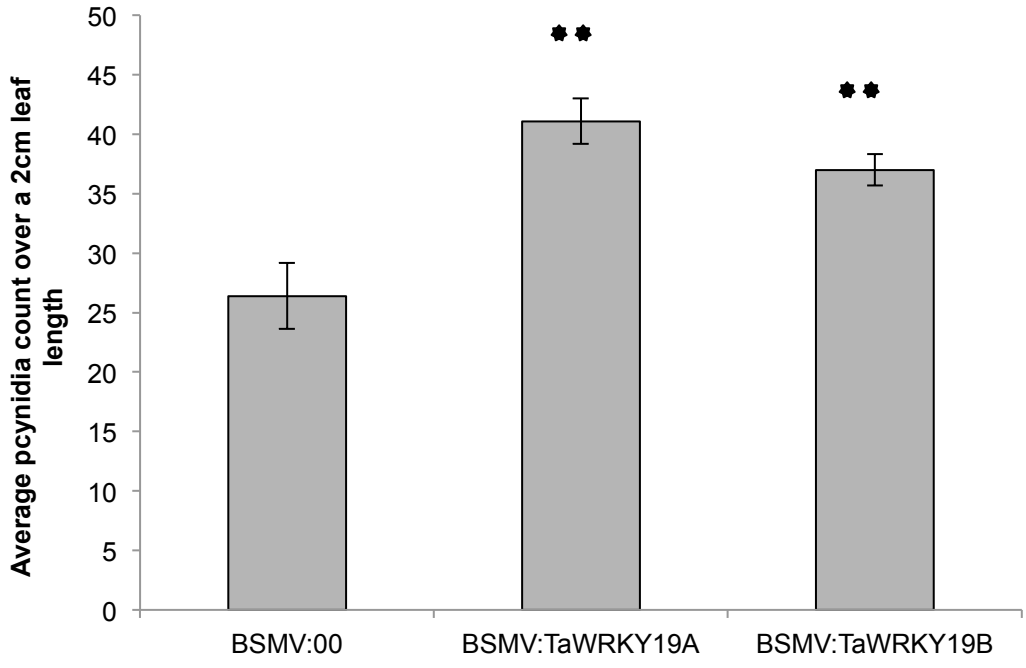

Figure 3.11: Pycnidia count from Septoria infected, TaWRKY19 silenced wheat leaves. Pycnidia were counted over a 2cm leaf length per leaf. Error bars correspond to +/- 1 standard error. The experiment was repeated independently 3 times with 5 leaves per experiment counted. Double asterisks are used to denote a results difference from the control of $p<0.01$.

The leaves were then collected into 15ml centrifuge tubes (5 leaves per tube) and submerged in 10ml of deionised water. The spores were washed off by vortexing the tubes for 2 minutes, leaving for 3 hours and vortexing again for another 2 minutes. 10µl of this solution was then loaded onto a haemocytometer and counted under a light microscope, x20 magnification. Wheat with TaWRKY19 silenced shows an increase in Septoria sporulation compared to BSMV:00 silence wheat (figure 3.12), with a statistically significant increase of 66% and 60% for BSMV:TaWRKY19A and BSMV:TaWRKY19B respectively compared to the BSMV:00 control. Student T-tests comparing the BSMV:00 to BSMV:TaWRKY19A spore counts have a value of 0.0132, making it statistically significant with over 95% confidence. A students T-test comparing BSMV:00 to BSMV:TaWRKY19B spore counts have a value of 0.0185, showing a confidence of over 95%.

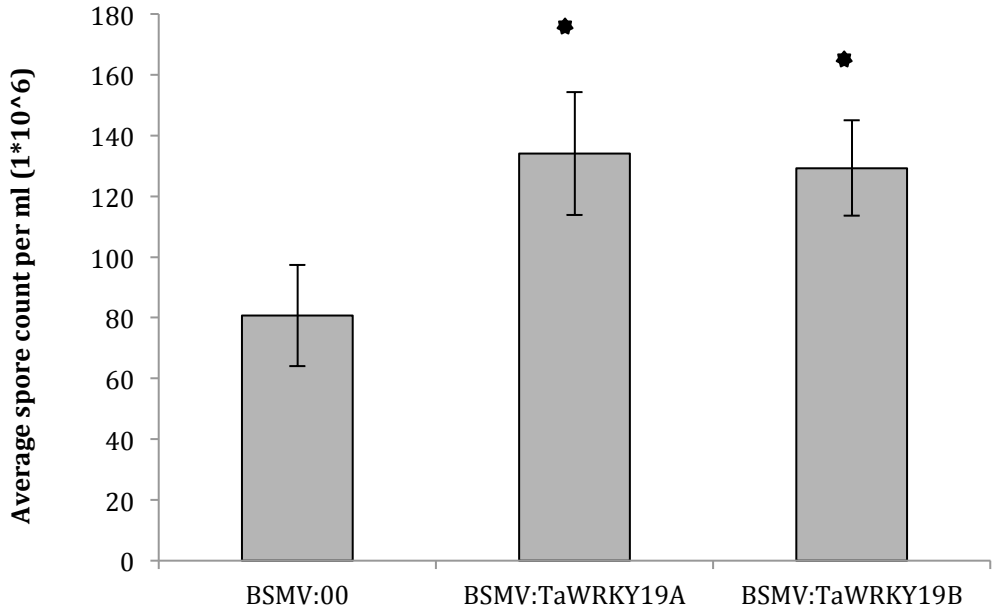

Figure 3.12: Septoria spore counts from TaWRKY19 silenced and infected leaves. Five leaves were submerged in 10ml of water and vortexed to wash the spores from the pycnidia. Spores were then counted using a haemocytometer under a light microscope (x10 magnification). Four spore counts across independent 4x4 squares on the haemocytometer were performed per spore suspension. Error bars represent +/- 1 standard error. This experiment was repeated independently 3 times. Asterisks are used to denote a results difference from the control of $p<0.05$.

Considering VIGS treatment merely reduces gene expression (as opposed to a full knockout in which there is no target gene expression) the differences in the pycnidia and spore count may be higher if the gene was fully knocked out. Therefore I extrapolated the data to assess the effect of modulating the expression of TaWRKY19. This is important as different varieties may have different levels of WRKY19 gene expression therefore working out the level of expression could then be used to predict Septoria infection severity.
Per 1% of silencing the pycnidia count for BSMV:TaWRKY19A increases by 1.10% and the spore count increases by 1.20%. For BSMV:TaWRKY19B silenced wheat the pycnidia count increases by 0.93% and the spore count increases by 1.10% per 1% of silencing.

Through these silencing and infection experiments I can now say that TaWRKY19 has a role in defence, acting as a positive quantitative regulator.

3.6 TaWRKY19 RNA sequencing

Towards the end of the project RNA sequencing was performed using the TaWRKY19 and BSMV:00 (control) silenced wheat lines, with and without Septoria infection. The aim was to identify genes whose expression changed in TaWRKY19 silenced plants, and to see if this changes post infection. Three RNA samples from each of the four conditions were used. RNA from BSMV:TaWRKY19B treated plants were used due to the higher levels of silencing (figure 3.9). Infected samples 12dpi were chosen due to the timing of the switch from biotrophic to necrotrophic growth and it being the time point in which TaWRKY19s expression is high (figure 3.2).

The RNA samples were tested for their quality, libraries prepared and run by the sequencing laboratory in Department of Biosciences, University of Durham. The data from the RNA sequencing was then mapped and aligned to the most up to date version of the wheat genome by KWS (Einbeck). I was advised that the results from this experiment were preliminary, with extra quality control checks needed. A heatmap comparing the samples is shown in supplemental figure 2. It shows a clear expression difference between the healthy and Septoria infected wheat in both the control and TaWRKY19 silenced plants, although it should be noted one of the control Septoria infected samples does cluster with the uninfected samples.

The most differentially expressed genes between BSMV:00 and BSMV:TaWRKY19B silenced plants were calculated (by KWS). 120 genes were downregulated strongly (factor of two difference or more) in TaWRKY19 silenced wheat. More genes were upregulated in TaWRKY19 silenced wheat, with 210 showing a differential expression of higher than a factor of two.

Comparing the uninfected samples to the infected samples, there are 172 genes downregulated and 356 genes upregulated (by more than a factor of two). Unfortunately other comparisons (for instance looking at genes that are differentially regulated in TaWRKY19 silenced plants between uninfected

and infected samples) were not performed. Further work looking at these data sets needs to be performed in order to identify specific genes and gene families whose expression changes between the treatments (supplemental table 2 and 3). This may help to identify how silencing TaWRKY19 leads to increased Septoria infection.

3.7 TaWRKY19 localisation

Now it has been confirmed that TaWRKY19 does indeed have a role in wheat's defence response against Septoria, the next step was to try and further describe the mechanism behind its role in defence response.
This first involved cloning the full length CDS of TaWRKY19. TaWRKY19 is 1,407bp long (figure 3.4). Primers were designed to amplify from the ATG until the TAG stop codon. PCR using Q5 proof reading DNA polymerase enzyme was used to clone TaWRKY19. Multiple different PCR conditions were tested before a band of the correct size was produced, including changing cDNAs, annealing temperatures, addition of DMSO and betaine. In the end touchdown PCR was employed with an extension time of 1 minute. From a previously published paper[152] and RNA sequencing[153] it is known that TaWRKY19 is also upregulated in abiotic stress, such as cold stress (24-24 hours)[152]. Therefore I put wheat seedlings (2-weeks-old) into cold conditions (4^0C) for 24 hours and collected leaf samples for RNA extraction and cDNA synthesis. I had also previously collected samples from wheat roots and processed them into cDNA. As seen in figure 3.2, TaWRKY19 is upregulated after Septoria infection; I chose 12dpi cDNA, as this sample had a high level of expression. To ensure one sample showed high levels of TaWRKY19 expression I used all three cDNA samples to try and amplify the CDS, this is shown in figure 3.13. These cDNA concentrations had not been amended to ensure they were the same so no assumptions can be made about expression levels in each of the cDNAs. All 3 cDNA samples show a band of the right size and so all three bands were collected for gel extraction and D-TOPO cloning procedure.

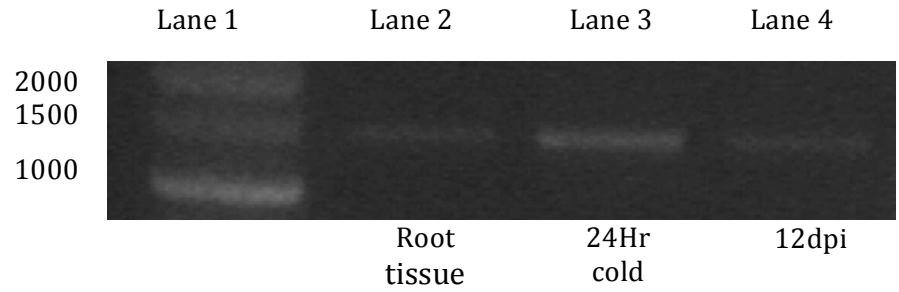

Figure 3.13: Gel of cloning PCR for TaWRKY19 CDS. PCR products were run on a 0.8% agarose gel in 1xTAE buffer for size separation and visualised under UV light with quantity one software. Hyperladder 1kb plus was used to assess DNA fragment size (lane 1). Different cDNAs were used in the PCR to clone TaWRKY19. Primers were designed to clone TaWRKY19 from it's start codon until the stop codon (1,407bp).

After cloning the gel-extracted band into the D-TOPO entry vector the construct was transformed into *E. coli* and grown overnight on LB agar media plates containing kanamycin for selection. Colonies were tested using PCR with the M13 forward primer (which primes to a region on D-TOPO before the insertion site) and a gene specific reverse primer (which primes to an internal region before the stop codon). The PCR was then run on a 0.8% agarose gel for size separation. Positive colonies were selected as those who had a band around 1.29kb (1,152kb gene band + 140bp vector) (figure 3.14). All the colonies tested were positive for an insert. Two of these positive colonies were selected and grown overnight; these were colonies 1 and 3.

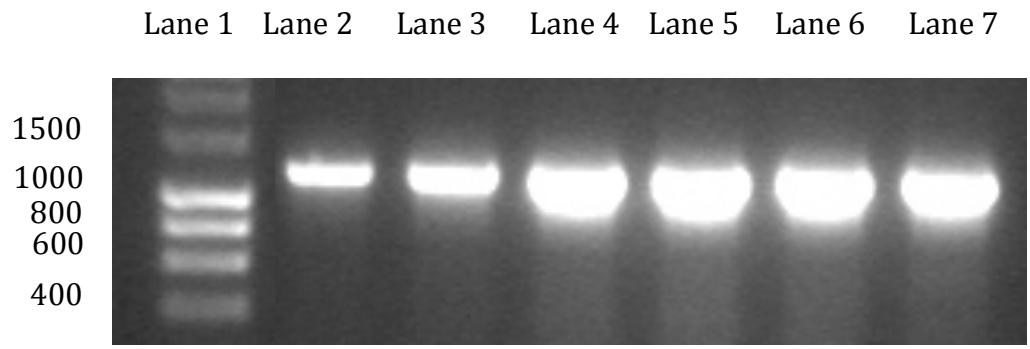

Figure 3.14: Gel of colony PCR for TaWRKY19 in D-TOPO entry vector. A PCR to test for successful TaWRKY19 cloning was run using M13 forward primer and a TaWRKY19 specific reverse primer. PCR products were run on a 0.8% agarose gel in 1xTAE buffer for size separation and visualised under UV light with quantity one software. A 1kb hyperladder was used for size indication (lane 1). The expected size for positive cloning of TaWRKY19 into the entry vector D-TOPO was 1,290bp.

The overnight cultures were then used for plasmid extraction and sent for sequencing using the M13 F and R primers. A single clone containing the correctly sequenced CDS was selected. D-TOPO is an entry vector; the destination vector chosen for this experiment was pEARLEYGATE104. This is because the localisation of TaWRKY19 was to be studied within the plant cell through confocal microscopy. pEARLEYGATE104 uses the 35S promoter to express TaWRKY19. I first had to use MluI restriction enzyme to disable the kanamycin resistance within the D-TOPO construct. This enzyme cuts the D-TOPO vector twice disabling it, it does not cut within TaWRKY19s CDS. Once cut, I ran the product on a 0.8% agarose gel, excising a fragment of 3,055bp (1407bp + 1,648bp)before gel extracting the DNA. The product was then put into an LR reaction (which facilitates the transfer of TaWRKY19 CDS from the entry vector to the destination) along with LR clonase and pEARLERYGATE104 destination vector. This was then transformed into *E. coli* DH5α cells and grown overnight on LB agar plates with kanamycin selection. Again colonies were tested for positive transformation using PCR

with YFP forward primers and a gene specific reverse primer. A positive colony was chosen to grow up and transform into *A. tumefaciens*.

It was expected that TaWRKY19 would localise to the nucleus being a TF but I decided to check to see if it localised elsewhere, which would potentially indicate a method of regulation. Also the idea was to potentially use this system to study whether introducing a pathogen or other stress causes a change in the localisation of TaWRKY19. This would add weight to current evidence that TaWRKY19 is involved in defence. Other WRKY genes have been shown to localise to the nucleus[154,155]. AtWRKY40s localisation was found to change after treatment with abscisic acid (ABA), but not after treatment with NaCl, flg22 or MeJA[155].

A. tumefaciens expressing P19 (which supresses silencing within the plants), YFP and YFP:TaWRKY19 were grown up overnight and then resuspended in 10mM $MgCl_2$ until an 0.4 O.D.600. 0.1nM of acetosyringone was then added to the mixtures and incubated for 2-3 hours. Two mixes of 1:1 P19:YFP and 1:1 P19:YFP:TaWRKY19 were prepared and infiltrated into the middle leaves of 4-week-old *N. benthamiana* plants. The plants were allowed to grow for 3 days before being used for experiment. The middle leaves were chosen as they are fairly easy to infiltrate, unlike the newest leaves, whilst also having higher levels of expression than the oldest leaves.

Small sections of infiltrated leaf (0.3*0.3cm) were placed onto microscope slides and submerged in PP11 (perfluoroperhydrophenanthrene). P11 enters the plant leaf pushing the air pockets out of the mesophyll spaces. It also has a refractive index similar to the plant cells, leading to clearer pictures, deeper into the plant tissue[156].

As can be seen in figure 3.15 YFP localises all throughout the cell, as expected, whereas TaWRKY19 only localises to the nucleus. This is not unsurprising being a TF. I only repeated this experiment once, however another published paper has also looked into the localisation of TaWRKY19 and found the same results[152]. If I were to do it again I would also ensure I used dyes that stain cellular components, such as DAPI that stains the cell nucleus.

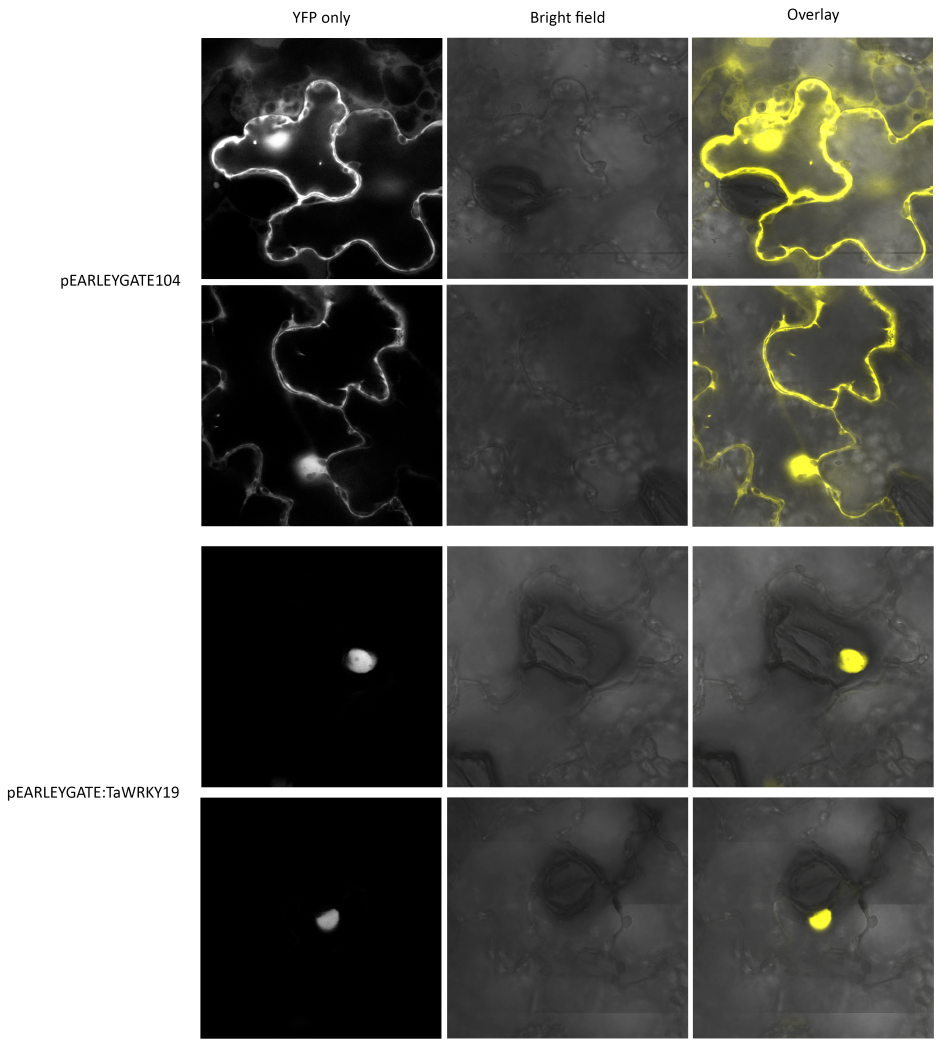

Figure 3.15: Localisation of TaWRKY19. *A. tumefaciens* expressing P19, YFP and YFP:TaWRKY19 were diluted in 10mM $MgCl_2$ to an O.D. 600 of 0.4. Four-week-old *N. benthamiana* plants were infiltrated with equal amounts of P19:YFP and P19:YFP:TaWRKY19 and allowed to grow 3 days. An SP5 confocal microscope was used to visualise YFP and YFP:TaWRKY19 within the leaf. A. YFP only filter. B. Bright field. C. YFP and bright field overlap.

3.7 TaWRKY promoter

The first step was to identify TaWRKY19's promoter region. Multiple databases were used (KWS's internal and Earlham Institute[151]) to find the region upstream of the ATG start codon of TaWRKY19. Unfortunately I could not find a previously identified TaWRKY19 promoter so the plan was to clone

2kb upstream of the ATG start codon. Although the wheat genome has been sequenced it had yet to be fully assembled (at the time of writing). Therefore there are some stretches of the genome that are not completely annotated; N's represent these sections in the database. Unfortunately 1497bp upstream (Supplemental figure 3) of the ATG start codon there was a run in which the genome was not fully assembled, hence I could only obtain the promoter sequence data for 1497bp.

I then used an online database (PlantPAN[157,158]) to analyse the promoter, allowing the prediction of potential TF binding sites. These are based on previously studied TF/promoter interactions in Arabidopsis, Rice and Maize. The results are summarised in table 3.4, each hit represents a unique TF (from Arabidopsis, rice and maize) that binds to a region in the promoter of TaWRKY19 (based on known binding sites for each of the TFs). Some TFs have multiple binding sites over the promoter. The top predicted hit is TFs from the AP2/ERF family, which are involved in the regulation of a broad range of stresses[159]. In wheat there are predicted to be 117 AP2 genes, with 47 of these in the AP2/ERF subfamily. This is less than in Arabidopsis and rice (147 and 164 total and 65 and 79 AP2/ERF subfamily respectively)[160]. The lower number of identified wheat genes may increase once the genome has been fully assembled and reanalysed. They are most highly expressed in the wheat leaves (18% of AP2/ERFs identified)[160], this is interesting based on the nature of Septoria's infection process.

The next family highly represented is the bZIP family; this family were highlighted in the original microarray experiment[138] as one of the families that had large changes in expression after Septoria infection. Interestingly only one WRKY binding site was predicted.

Family	No. of TFs
AP2;ERF	62
bZIP	30
Dof	18
NAC;NAM	16
TCP	11
AP2;B3;RAV	10
AP2;RAV;B3	10
bHLH	9
GATA;tify	9
AT-Hook	8
Others	8
Homeodomain;HD-ZIP	6
B3	5
Homeodomain;TALE	5
MADF	5
Myb/SANT	5
Myb/SANT;MYB	5
Myb/SANT;MYB-related	5
Myb/SANT;trp;MYB	5
SBP	4
Storekeeper	4
B3;ARF	3
CG-1;CAMTA	3
Alpha-amylase	2
AP2	2
C2H2	2
FAR1	2
GATA	2
Homeodomain;bZIP;HD-ZIP	2
LEA_5	2
MADF;Trihelix	2
MYB-related	2
MYB;ARR-B	2
Myb/SANT;MYB;ARR-B	2
TCR;CPP	2
BES1	1
bZIP;B3	1
Dehydrin	1
E2F	1
E2F/DP	1
E2F/DP;E2F	1
EIN3;EIL	1
HD-ZIP	1
Homeodomain;bZIP;HD-ZIP;WOX	1
LFY	1
LOB;LBD	1
MADS box;MIKC;M-type	1
MYB	1
Myb/SANT;trp;MYB;NF-YC	1
NF-YB;NF-YA;NF-YC	1
TBP	1
Trihelix	1
VOZ	1
WRKY	1
ERF	1
NAC	1

Table 3.4: List of predicted TFs that bind to TaWRKY19 promoter. DNA sequence for TaWRKY19 promoter was input into PlantPAN software [157,158]. TFs from the plant species Arabidopsis, rice and maize were selected for the analysis. Binding sites for the TFs are in supplemental table 3.

Primers were designed to amplify from the beginning of the identified promoter region until just before the ATG start codon of TaWRKY19. At the 5' end of the forward and reverse primer restriction enzyme sites for XmaI and XbaI were included respectively. These sites are needed for cloning into PTUY1H destination vector, which is used for the Y1H experiment. Wheat DNA was extracted for use as a template in cloning the promoter. Multiple PCR conditions were tested before a band of the correct size was seen (some of these can be seen in figure 3.16). The final PCR conditions that produced the promoter band were an annealing temperature of 52^{0}C, HiFi polymerase and an extension time of 2:15 minutes with the addition of DMSO and 30 PCR cycles. I also had to perform a nested PCR, diluting the first PCR product 1/10 in water, using this mix as the template for the second PCR. The PCRs were then run on a 0.8% gel (figure 3.16). As can be seen, the addition of betaine and DMSO+betaine to the PCR did not produce any amplified products in either of the PCRs. However the addition of DMSO led to a band in both the first PCR and the nested PCR, with a band size reduction corresponding to the size difference of the amplicon.

The DNA from the band highlighted in figure 3.16 (nested PCR) was extracted and cloned into pJET1.2 (for blunt ended PCR fragments), before being transformed into DH5α *E. coli* cells and left to grow overnight on LB agar with ampicillin for selection. PCR using a pJET specific sequencing primer and a promoter specific reverse primer were used to test the colonies for positively cloned colonies (similar to the previously shown colony PCR in figure 3.14). Two of the positive colonies were then grown up and plasmid DNA extracted to check the DNA sequence, the sequencing reactions used forward and reverse pJET specific sequencing primers. Once the sequence had been confirmed the next step was to clone the promoter into PTUY1H[133] vector using restriction enzyme cloning (XmaI and XbaI). The PTUY1H vector and pJET with TaWRKY19 promoter were both incubated with XmaI and XbaI overnight (cutsmart buffer) and run on a 0.8% agarose gel. Fragments around the size of 1,409kb and 6,500kb were isolated from the gel for TaWRKY19 promoter and PTUY1H vector respectively. These fragments

were extracted from the gel using a gel extraction kit before being ligated together overnight with T4 DNA ligase. The resulting ligation product was transformed into DH5α *E. coli* cells and grown up overnight on LB agar plates with kanamycin for resistance selection. The colonies were again screened for PTUY1H vectors containing the TaWRKY19 promoter. This was done using PCR with a forward primer specific to PTUY1H and a reverse primer against TaWRKY19 promoter. One of the positive colonies was grown up overnight and the plasmid isolated from the resulting culture. The final step was to transform this vector into Y187α yeast ready for the yeast 1 hybrid assay (chapter 4).

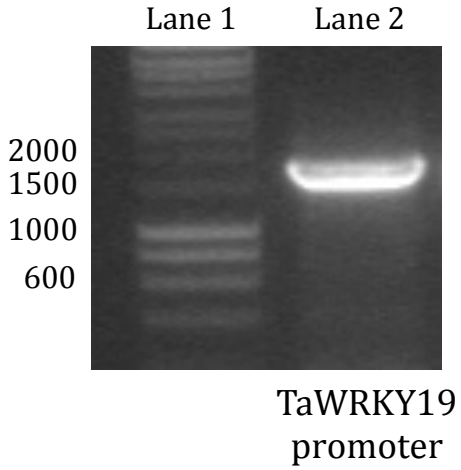

Figure 3.16: Gel of TaWRKY19 promoter cloning PCR. PCR products were run on a 0.8% agarose gel for size separation and visualized under UV light with quantity one software. A 1kb hyperladder was used for size indication (lane 1). The expected size for TaWRKY19 promoter (lane 2) was 1,409kb.

3.8 Conclusion

The aim of this chapter was to identify WRKY TFs that are involved in wheat defence against Septoria.

WRKY TFs were focused upon based on previous microarray experiments looking for changes in expression after Septoria infection. The initial screening focussed on 15 TaWRKYs that were homologues of previously published WRKY genes from Arabidopsis and rice. Healthy and Septoria

infected time courses were used to investigate the expression profiles of the 15 TaWRKYs. From this four TaWRKYs were found that were all upregulated after Septoria infected. After initial experiments the list was again narrowed down to two TaWRKYs – TaWRKY19 and TaWRKY9. The focus of this chapter was TaWRKY19. It is worth noting that although TaWRKY2 was upregulated during initial infection assays it's silencing did not affect wheat's ability to defend against Septoria either positively or negatively[161]. This made for a good negative control, showing that although some WRKYs expression changes after infection, not all WRKYs are involved in defence. It also shows that the process of silencing WRKYs does not always lead to a change in Septoria infection.

Through RACE experiments the 3' UTR was sequenced, allowing for generation of 2 specific VIGS fragments. Average silencing levels of 38% and 45% were achieved for BSMV:TaWRKY19A and BSMV:TaWRKY19B. Whilst these may not be as high as previously published the small modulation in TaWRKY19 expression still led to wheat becoming more susceptible to Septoria. Ideally silencing levels would have been studied when TaWRKY19's expression was highest between 8-14dpi. This is when I assume TaWRKY19 silencing would have the most effect however that was, unfortunately, an after thought and not considered at the time. The most accurate way of measuring silencing levels would be to measure the amount of TaWRKY19 protein translated. Protein levels could be measured by generating an anti-body against TaWRKY19, however this can be a difficult process so was not undertaken during this project.

Wheat lines silenced with TaWRKY19 did show an increase in Septoria infection, with symptoms beginning a day earlier, an increase in pycnidia and spores. Although these increases may not be as drastic as previously published VIGS/Septoria infection work there is still a statistically significant difference that may be useful for breeders. This is particularly true when looking at work done previously on TaWRKY19, in which overexpresing TaWRKY19 in Arabidopsis led to increased tolerance to osmotic/dehydration, salt and freezing stresses[90,91]. A breeding target that can offer an increase in abiotic and biotic stresses must be of significant

benefit. It is also the first case in our laboratory of gene silencing leading to an increase in Septoria infection and therefore identification of a positive regulator of defence. Work presented in this chapter based on TaWRKY19 silencing and Septoria infection led to TaWRKY19 being patented for use in resistance variety wheat breeding in collaboration with KWS[162].

TaWRKY19 is located in the nucleus of the cell. However this is under the control of a 35S promoter and in 'normal' conditions. There are examples of TFs subcellular localisation changing under stress responses[163,164]. Further repeats and experiments looking into the localisation during infection may result in interesting insights into the regulation of TaWRKY19.

To begin building up a network of defence against Septoria I decided to go upstream of TaWRKY19. This was in the hope of finding multiple breeding targets that can be stacked to reduce the likeliness of Septoria evolving resistance as has happened before. The second aim was to find a target that was further up the chain, closer to the perception of the pathogen. These TFs may have more control over defence related genes, leading to a more pronounced effect when silenced and infected with Septoria. The first step was to clone the promoter of TaWKRY19. The sequence 1,493bp upstream of TaWRKY19's start codon was identified. I proceeded to amplify this region using PCR with DMSO as a PCR additive, and clone the promoter into the Y1H bait vector PTUY1H ready for the Y1H experiment (chapter 4).

4. TabZIP2 binds TaWRKY19 promoter and acts as a susceptibility factor against Septoria

4.1 Introduction

Yeast 1 hybrid (Y1H) assays allow protein-DNA interactions to be tested in the context of the nucleus[165]. In this case, the interaction of TFs on promoter sequences although other DNA binding proteins lacking transcriptional activating domains can also be tested. Other advantages of a Y1H screen include the ability to study many TFs/DNA interactions and the speed of the process[166].

The DNA promoter region (bait) is cloned upstream of the GAL4 promoter and reporter gene (HIS3) into a vector that contains a leucine selection marker. The vector is then transformed into Y187α yeast strain (figure 4.1). Positively transformed yeast cells can be selected for by growing on minimal Synthetic Defined (SD) base media with all the amino acids minus leucine. The TFs (prey) are cloned into a vector upstream of a GAL activating domain (GALAD) into a vector containing a tryptophan selection marker. The vector is then transformed into AH109A yeast (figure 4.1). Selection is similar to that of the promoter containing yeast except with an amino acid mixture lacking tryptophan. Having the GALAD domain allows for DNA binding proteins that do not activate transcription to be tested, such as chromatin remodelling and DNA repair proteins. Having transformed the yeast (of opposing mating strains) they are next mated, bringing the prey and bait together. If the TF binds to the DNA bait in vivo the GALAD will come into contact with the GAL promoter, leading to transcriptional activation of the HIS3 reporter gene (figure 4.1). HIS3 is a imidazoleglycerol-phosphate dehydratase involved in the 6th step of histadine synthesis[167]. Activation of HIS3 allows the yeast to grow on triple drop out media (-L, -W and –H), indicating to the experimenter a positive interaction. However the system can be leaky, with yeast transcription factors binding to the DNA bait domain, therefore 3-amino-trizole (3AT) in increasing concentrations is included to test the strength of the interactions. 3AT is a competitive inhibitor of the HIS3 enzyme, hence if there is a small amount of activation by yeast TFs or a

weak binding of the prey to the bait promoter growth will only happen on media containing no or low concentrations of 3AT. One thing to note is that Y1H experiments do not determine whether the TF will be a positive or negative regulator of the promoter region to be tested[133,166,168-170]. Y1H screens also will not pick up any TFs that need to be post translationally modified or bind as homo/heterodimers[171]. There can also be false-positives interactions identified even with the selection pressures, meaning further binding experiments may need to be performed[166].

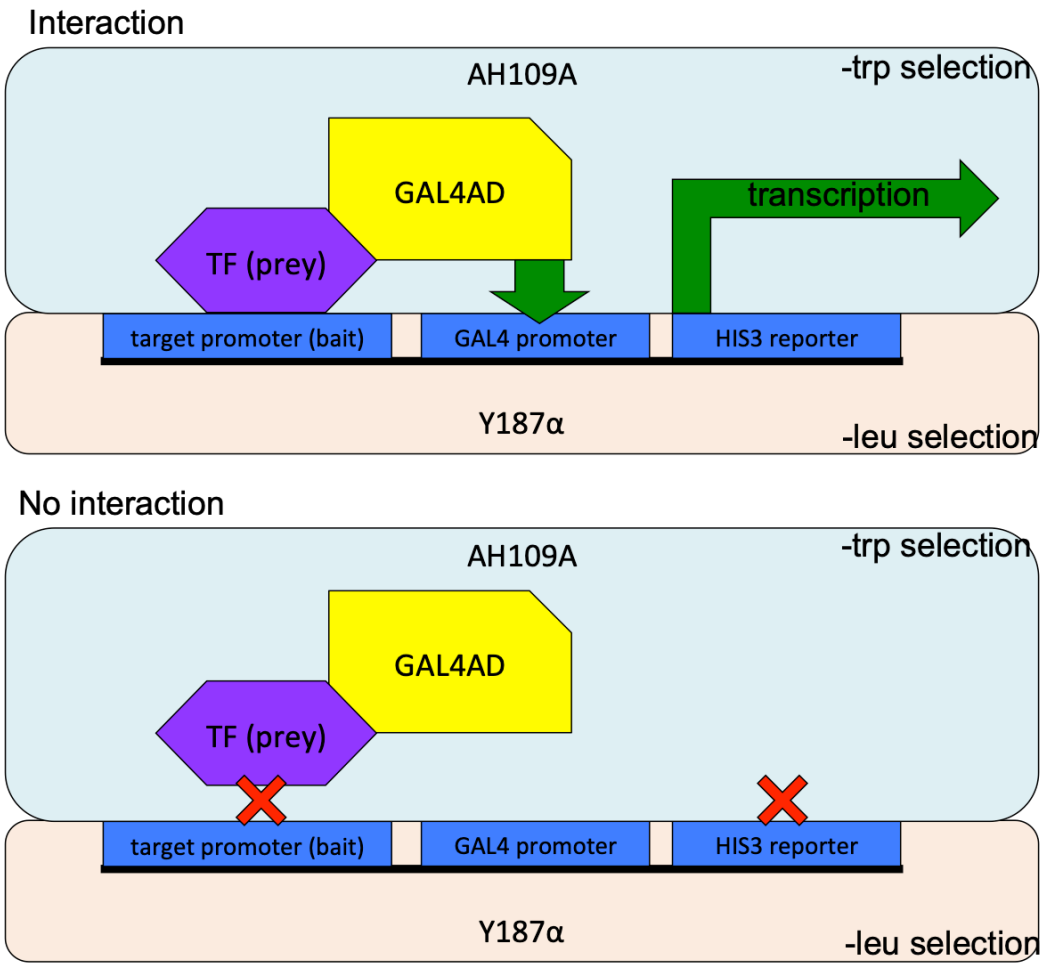

Figure 4.1: Yeast 1 Hybrid schematic. The top panel shows a positive interaction leading to HIS3 reporter gene transcription. The bottom panel shows no interaction between TF and target promoter, leading to no HIS3 transcription. Figure modified from [170]

Basic (DNA binding region) leucine zipper (bZIP) transcription factors are a highly diverse family, which, unlike WRKYs, are found throughout the eukaryotic kingdom. The basic (N-X_7-R/K) and leucine zipper (L-X_6-L-X_6-L)

regions form two adjacent alpha helixes (X_9 separating the two domains), with two bZIPs interacting (through the leucine zipper) to bind DNA (through the basic region) in a zipper like action[172]. As they primarily function in dimers through the leucine zipper[173], many different dimer combinations can be formed allowing high levels of regulatory flexibility[174]. In Arabidopsis and wheat there are 78 and 187 identified bZIPs respectively[172,174,175], which are classified into 13 groups depending on their bZIP and other domains structure[174]. The largest class is the S class, the most studied of which are in the S_1 subdivision. These bZIPs are noted by their small size (roughly 20kDa) containing the bZIP domain, with an extended leucine zipper, and a lack of introns. This class have been found to form dimers with the C class, which are similar in structure.

Plant bZIP TFs tend to bind to DNA promoter regions with a core ACGT, such as A-box (TACGTA), C-box (GACGTC) and G-box (CACGTG), with different bZIPs having different affinities for the different boxes[176].

Both WRKY and bZIP transcription factors have been shown to be involved in a wide range of biological processes, including pathogen defence.

4.2 Arabidopsis TF library for yeast 1 hybrid

Castrillo and Turck et al[133] generated a library of Arabidopsis TFs, adding to a previous library (REGIA project[177]). In total their library has around 1,200 unique TFs. They also generated a vector, PTUY1H, for cloning promoter sequences into for use in the Y1Hs. The library can be purchased from the Nottingham Arabidopsis Stock Centre (NASC), who sends glycerol stocks of the TFs in yeast (AH109A) on 15 separate 96 well plates. Within in Castrillo and Turck et al[133] paper they have also conveniently written an easy to follow protocol for the mating of the bait promoter containing yeast with the library, including time frames for each step.

My supervisor (Ari Sadanandom) decided that the use of this library to find TFs that bind to TaWRKY19's promoter would be a useful next step for the project. The idea was to find other TFs that may be involved in defence against Septoria. Using a TF library allows for many interactions to be tested

rapidly[166] and it was readily available as it had previously been used in our laboratory.

The first step was to revive the library onto minimal SD base media with –W amino acid mixture. This was performed using a 96-pin replicator that transfers 5µl of liquid on the end of each pin to allow transfer onto the new plates. These were then grown at 28^{0}C for 3 days. As not all the colonies initially grew, a second set of plates was stamped out and used to fill any gaps from the first set. Even between the two sets not all of the wells grew. Figure 4.2 shows a cartoon representation of which wells grew (highlighted in grey). The figure also has additional information I will discuss later. Overall 68% of the library grew, with some plates growing better than others. Two plates contain the majority of the WRKY TFs, these are plate 4 (16 WRKYs) and plate 10 (43 WRKYs). Whilst plate 4's revival was fairly successful (86% coverage), plate 10's was not, with only 17% coverage. By far the worst revival was of plate 14, where only 2 of the 96 wells grew. There are a few families represented on this plate including (but not limited to) AP2/ERF (12 wells), bZIP (3 wells), NAC (11 wells) and bHLH (27 wells), which are in the top 10 hits from the plantPAN promoter predication software (table 3.4). However, between the plates, there is still a good coverage of TFs from each of the families.

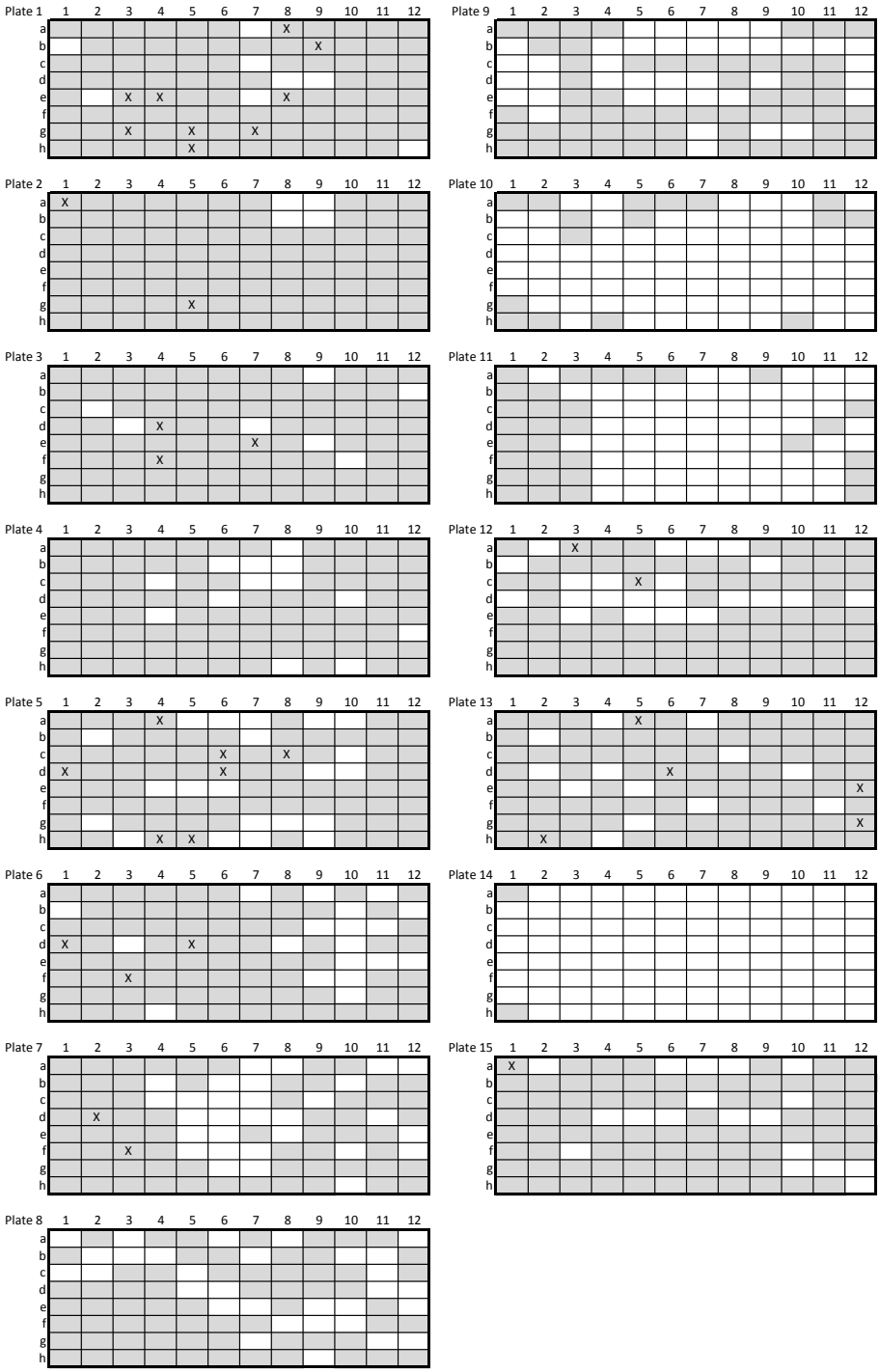

Figure 4.2: Y1H TF library revival and TaWRKY19 promoter binders. A representation of the fifteen 96-well plates from the Arabidopsis Y1H library[133] (approx. 1,200 different TFs) showing the wells that were successfully revived from glycerol stock (highlighted in grey). Wells marked with an X represent the TFs that bound to TaWRKY19 promoter identified through a Y1H experiment grown upon minimal SD base media with amino mix –L-W-H + 20mM 3AT for selection.

4.3 Positive interactions between Arabidopsis TFs and TaWRKY19 promoter

After reviving the TF library, the next step was to mate it with TaWRKY19 promoter (in PTUY1H vector, Y187α yeast strain). To do this a large culture of YPAD, inoculated with AH109A expressing PTYU1H:TaWRKY19 promoter construct was grown overnight at 28^{0}C. Simultaneously the TF yeast library was inoculated into fifteen 96-well plates containing YPAD liquid media before being grown overnight at 28^{0}C. The next step was to mate the two yeast strains. To do this each well of the TF library was inoculated with a set amount (100µl) of the TaWRKY19 promoter construct yeast and incubated for 48 hours at 28^{0}C. Using a 96-pin replicator fresh media (SD minimal base with –L-W amino acid mixture) was inoculated with the previous cultures before being grown for 24 hours at 28^{0}C. This step was performed for selection of the mated yeast cultures.

To determine the concentration of 3AT needed for the final selection phase of the experiment, Y187α yeast containing TaWRKY19 promoter in PTUY1H with mated with AH109A yeast containing pDEST22. The TFs in the Y1H library are all in pDEST22 vector, hence its use as a control. It is regularly used in both Y1H and Y2H hybrid experiments. After the mating process the resulting cultures out spread onto minimal SD base agar media plates with –L-W amino acid mix (to ensure mating), -L-W-H amino acid mix and –L-W-H amino acid mix with increasing amounts of 3AT (10, 20, 40, 60, 80 and 100mM). From this experiment it was determined that using 20mM of 3AT was enough to negate any residual activation of the HIS3 reporter gene by yeast's own TFs (data not shown).

Now the 3AT concentration had been worked out, the final step in the Y1H library assay was to stamp out the mated library+TaWRKY19 promoter onto selection media. This involved using the 96-pin replicator to stamp out each of the wells onto 3 different sets of minimal SD base media plates containing –L-W amino acid mix, -L-W-H amino acid mix and –L-W-H amino acid mix + 20mM 3AT. The plates were then grown at 28^{0}C for 3 days, with pictures taken after this time to enable me to analyse the growth.

Figure 4.2 shows a cartoon representation of the 15 96-well plates. Highlighted in grey are the colonies that grew on the initial revival. Wells with an X represent those that grew on each of the selection medias, showing successful mating and a positive TF/TaWRKY19 promoter interaction. Of the 974 revived TF wells (68%), 34 grew on all three selection media. This represents 3.5% of the successfully revived wells.

The next step was to test the strength of the interactions between the TFs and TaWRKY19 promoter that grew during Y1H library screen. The 34 TFs were remated with TaWRKY19 promoter containing yeast, and mated yeast selected for using –L-W media (minimal SD base + amino mix). Three*5µls of each mated culture was pipetted onto plates containing minimal SD base with amino acids mix lacking –L-W, -L-W-H and-L-W-H + 3AT (20, 40, 60 80 and 100mM). These plates were grown at 28^{0}C for 3 days (supplemental figure 4). The 3AT concentrations were chosen for testing based on information gained by reading previously published papers[178] who used Y1H assays, including Castrillo and Turck et al[133]. Figure 4.3 shows a representation of the growth, and therefore binding, of the 34 TFs to TaWRKY19 promoter. Four of these transcription factors show strong binding, still growing on the highest concentration of 3AT (100mM). There are some other TFs that also bound strongly (up to 80mM 3AT), however due to time constraints I decided to focus upon the top four (highlighted in light grey, figure 4.3). Some of the colonies also did not mate in this experiment, however since I had four targets it was decided not to follow these up.

Well	Gene ID	Gene description	No.	-L-W	+20mM	+40mM	+60mM	+80mM	+100mM
p1 a8	At4g37940	MADS (AGL21)	1						
p1 b9	At5g51870	MADS (AGL71)	2						
p1 e3	At3g12890	CO-like (ASML2)	3						
p1 e4	At4g27900	CO-like	4						
p1 e8	At2g46790	CO-like (APRR9)	5						
p1 g3	At1g74410	C3HC4 (ATL24)	6						
p1 g5	At3g61550	C3HC4 (ATL68)	7						
p1 g7	At2g18670	C3HC4 (ATL56)	8						
p1 h5	At1g21960	C3HC4 (RING/U-Box)	9						
p2 a1	At5g49450	bZIP (bZIP1)	10						
p2 g5	At3g10480	NAC (NAC50)	11						
p3 d4	At1g50420	GRAS (SCL3)	12						
p3 e7	At2g45680	TCP (TCP9)	13						
p3 f4	At3g27010	TCP (TCP20)	14						
p5 a4	At5g05410	DREB (DREB2A)	15						
p5 c6	At3g12720	MYB (MYB67)	16						
p5 c8	At1g56160	MYB (MYB72)	17						
p5 d1	At2g01060	G2-like (PHL7)	18						
p5 d6	At3g13040	G2-like (PHL6)	19						
p5 h4	At3g11440	MYB (MYB65)	20						
p5 h5	At1g26780	MYB (MYB117)	21						
p6 d1	At5g43270	SBP (SPL2)	22						
p6 d5	At2g47070	SBP (SPL1)	23						
p6 f3	At3g07740	SWI/SNF (ADA2A)	24						
p7 d2	At4g37790	Homeobox (HAT22)	25						
p7 f3	At3g50260	AP2/ERF (DEAR1/CEJ1)	26						
p12 a3	At2g13150	bZIP (bZIP31)	27						
p12 c5	At2g32950	WD-40 (COP1)	28						
p13 c5	At1g28160	AP2/ERF (ERF87)	29						
p13 d6	At5g44210	AP2/ERF (ERF9)	30						
p13 e12	At5g60830	bZIP (bZIP70)	31						
p13 g12	At1g35460	bHLH (bHLH1)	32						
p13 h2	At1g66470	bHLH (RDH6)	33						
p15 a3	at4g36990	HSF (HSFB1/HSF4)	34						
-ve control		TaWRKY19 promoter	35						

Figure 4.3: A representation of 34 TFs binding onto TaWRKY19 promoter through a Y1H experiment, with increasing selection. 34 TFs, identified through the initial Y1H experiment, were re-mated with TaWRKY19 promoter yeast and grown in liquid culture overnight. 5µl of this overnight was pipetted onto plates with minimal SD base media with an amino acid mix of either –L-W, -L-W-H, -L-W-H +3AT (20, 40, 60, 80 and 100mM). The plates grown for 3 days at 28^0C. Yeast that grew are highlighted in dark grey. TFs that grew on each selection are highlighted in light grey.

Figure 4.4 shows the growth of the mated yeast from the selection plates. We (Ari Sadanandom and myself) also chose to include one TF that did not bind as strongly (up to 40mM) but was part of the bZIP family. We chose to continue studying this based on the original microarray experiment findings that members of the bZIP family show large expression changes post Septoria infection[138]. Also, of the 34 TFs that bound to TaWRKY19 promoter in the initial Y1H assay (figure 4.2 and 4.3), three were bZIPs so we wanted to study a member of this family. Finally, PlantPAN promoter analysis software predicted the bZIP TF family to be the second highest binders to TaWRKY19 promoter (table 3.4). So we wanted to follow up on one of these bZIP TFs. AtbZIP1 was chosen out of the four based on published papers showing it has a potential link to nitrogen regulation[179]. This is important, as there have been observations by KWS that crops treated with more nitrogen showed higher levels of Septoria infection. AtbZIP70 does bind the strongest (up to 80mM 3AT), however this TF has not been studied as much at the time, hence why AtbZIP1 was chosen, which binds up to 40mM 3AT and has been studied in multiple different papers[179-184]. Another consideration was that PostTranslational Modifications (PMTs) might increase the binding of AtbZIP1 to TaWRKY19 promoters, which has been seen in other bZIP TFs[185,186]. My laboratory specialises in PMTs, particularly SUMOylation which has been shown to change the binding capacity of TFs to DNA in plants[187,188].

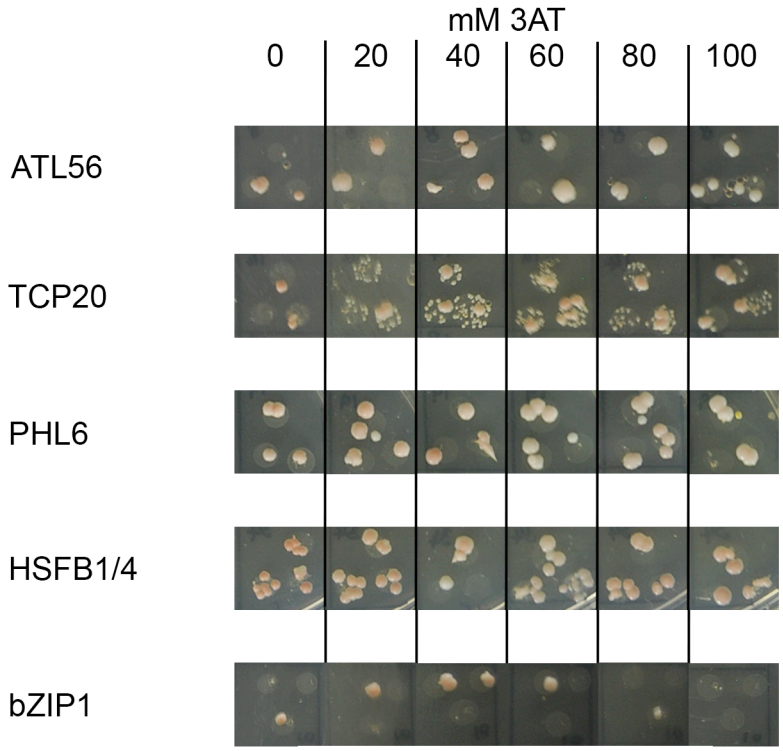

Figure 4.4: Y1H of strongest TFs binders to TaWRKY19 promoter. TFs that showed binding in the initial screen were re-mated with TaWRKY19 promoter and grown minimal SD base with amino mix –L-W-H and increasing 3AT selection (20mM, 40mM, 60mM, 80mM and 100mM). Colonies were grown at 28^{0}C for 3 days. Pictures of the 34 targets were taken, included in the figure are the five strongest TF binders to TaWRKY9 promoter.

As a secondary look the binding of another AtbZIP from the library, AtbZIP53, was also investigated. They are both S1 bZIP family members. It is closely related to AtbZIP1 (both DNA and protein sequence), showing similar modes of regulation that are not shared by other S1 bZIPs and also regulates a similar set of pathways and genes[189,190].

There is published evidence showing AtbZIP1 is involved in salt and drought stress[183,190] and is regulated in response to changes in nitrogen and light levels[179,191-193]. Due to AtbZIP1 and AtbZIP53s shared regulation and targets I decided to look back at the original library screen and if there was any

interaction. AtbZIP53 is represented in plate 12 well E03, as can be seen in figure 4.2 this well did not grow upon after revival from the glycerol stocks. Therefore primers were designed to clone AtbZIP53's CDS, amplifying from the start to stop codon.

I used PCR to amplify the CDS using the method as described in 2.4.2 (Q5 proof reading DNA polymerase PCR), with the following conditions for the variable steps; 55^{0}C annealing temperature, 20 seconds extension time and 30 cycles. I used cDNA from Col-0 wildtype plants. I then ran the PCR on a 1% agarose gel to separate the DNA bands by size (figure 4.5). AtbZIP53 CDS is 441bp long; a DNA band around this size can be seen in figure 4.5. I used both a 1kb ladder (lane 1) and 50bp ladder (lane 2) due to the small size expected, however the 50bp ladder is not very clear so I relied on only the 1kb ladder for size estimation. There are two bands (lanes 3 and 4) for AtbZIP53 due to the large PCR reaction volume not being able to fit into one well, so it was spread across two wells.

The gel extract was then cloned into p-DOPO as previously described in chapter 3, and cloned into the destination vector pDEST22 before being transformed into the yeast strain AH109A, using minimal SD media with amino acid mix –W for selection.

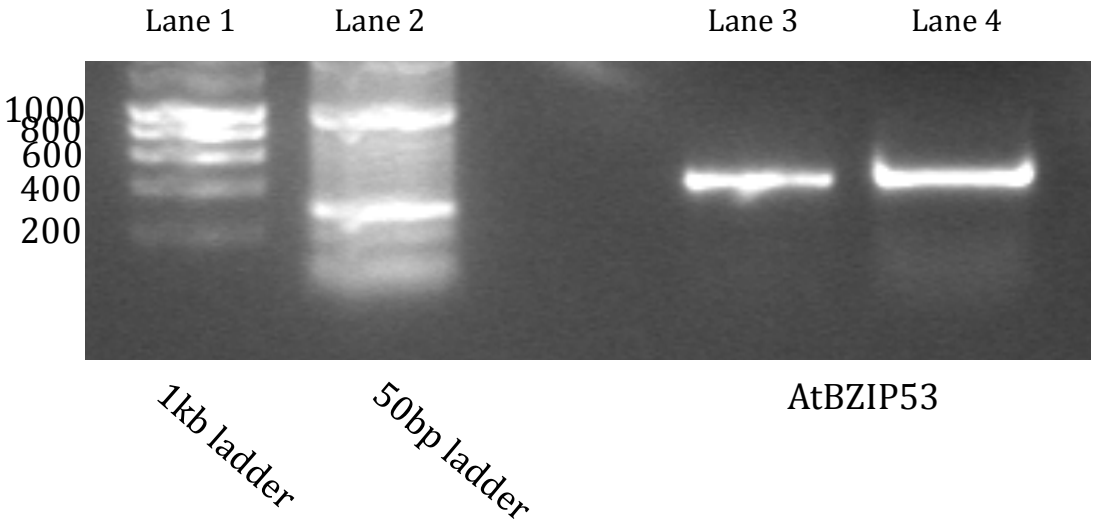

Figure 4.5: Gel of cloning PCR for AtbZIP53 CDS. PCR products were run on a 1% agarose gel in 1xTAE buffer for size separation and visualised under UV light with quantity one software. A 1kb and 50bp hyperladder were used for size indication (lane 1 and 2 respectively). The expected size for AtbZIP53 (lane 3 and 4) was 441bp.

The AtbZIP53 TF was mated with the TaWRKY19 promoter yeast to test for binding and binding affinity. The mated yeast was spread onto minimal SD base media with amino acid mix –L-W for selection. One colony was selected to grow up overnight in liquid culture before pipetting three 5µl spots onto the selection plates (figure 4.6). This is why I have not included a control –L-W column in this figure, as the colonies were all taken from a mated plate. In future experiments I would have included the controls. I also repeated the experiment with AtbZIP1 for binding strength comparison. AtbZIP53 binds to TaWRKY19 promoter much stronger than AtbZIP1, up to 100mM 3AT (figure 4.6). Again AtbZIP1 binds up to 40mM 3AT.

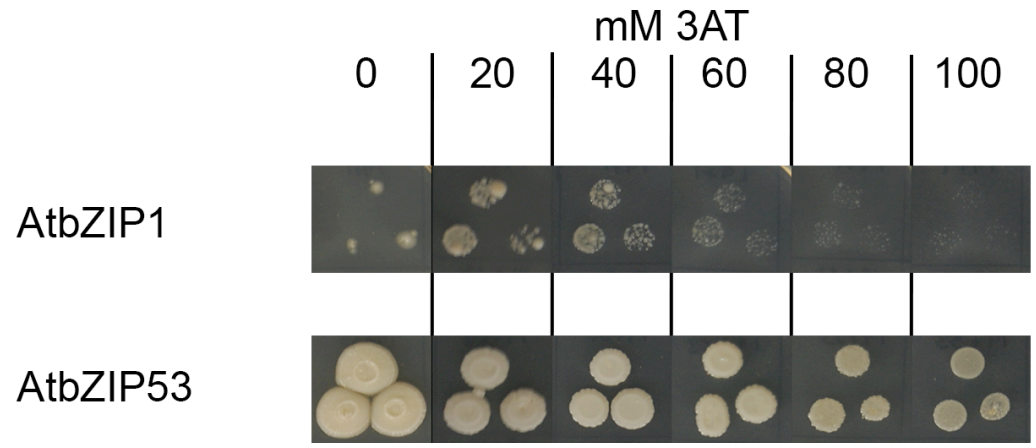

Figure 4.6: AtbZIP53 Y1H with TaWRKY19 promoter. AtbZIP1 and AtbZIP53 TFs mated with TaWRKY19 promoter and grown on increasing minimal SD base with amino mix –L-W-H and 3AT selection (20mM, 40mM, 60mM, 80mM and 100mM). Colonies were grown at 28^{0}C for 3 days before pictures were taken.

4.4 Identification of wheat homologues of targets

The TF library was made up of Arabidopsis TFs, therefore the next step was to find the wheat homologues for the final targets identified. To do this the blast tools on both the KWS databases and the publicly available database on EnsemblPlants[194] were used. An alignment of the closest wheat homologue for each of the six targets is seen in figure 4.7. The alignments were made using Clustal Omega software[195,196]. Papers identifying bZIP[175] and HSF[197] families in wheat have been published. Therefore I adopted their nomenclature for the wheat homologues of AtbZIP1, AtbZIP53 and AtHSFB1/4. Although some MYB-like genes have been identified in wheat the homologue of AtPHL6 has not so therefore was numbered after the Arabidopsis homologue. For both TCP and RING C2H2 TF families I could not find a definitive list and numbering, so again it was numbered after the Arabidopsis homologue (table 4.1). The identity score and expect value for each of the homologues can be seen in table 4.1. Identity scores are the percentage of amino acids in each position that are the same in both sequences. Expected values calculate the likelihood of an alignment occurring

by chance elsewhere in a genome of a set size, the smaller the number, the more homology.

A.

```
AtbZIP1       ------------MANAEKTSSGSDIDEKKRKRKLSNRESARRSRLKKQKLMEDTIHEISS      48
TabZIP2A      MSSPSRRSSSPESNIDGGSGSGSAGDERKRKRMLSNRESARRSRARKQQRMEELIAEASR      60
                          :.***  **:**** ********** :**: **: * * *

AtbZIP1       LERRIKENSERCRAVKQRLDSVETENAGLRSEKIWLSSYVSDLENMIATTSLTLTQSGGG      108
TabZIP2A      LQAENKRVEAQIGAYTTELTKVDGENAVLRARHGELAGRLQALGGVLE-----IFQVAGA      115
              *: . *. . :  * . .* .*: *** **:.:  *:. :. * .::      : * .*.

AtbZIP1       DCVDDQNANAGIAVGDCRRTPWKLSCGS-------LQPMASFKT      145
TabZIP2A      -PVDI------PEIPDPLLRPWQSPFAPQLATAGGMPDAFQF--      150
                **         : *    **:   .        :    .*
```

B.

```
AtbZIP53      MGSLQMQ------TSPESDNDPRYATVTDERKRKRMISNRESARRSRMRKQKQLGDLINE      54
TabZIP98A     MSSSSLSPGGGRLSGSDGDSGAT-FAAGDNRREKRRLSNRESARRSRLRKQQHLDELVQE      59
              *.* .:.      :. :.*..    :. *:*:.** :**********:***::*.:*::*

AtbZIP53      VTLLKNDNAKITEQVDEASKKYIEMESKNNVLRAQASELTDRLRSLNSVLEMVEEISGQA      114
TabZIP98A     VARLKAENARVLARANDITGQFVRVDQENTVLRARAAELGDRLRSVNQVLRVVEEFSGVA      119
              *: ** :**::  :.:: : :::.::.:*.****:*:** *****:*.**.:***:** *

AtbZIP53      LDIPEI---PESMQNPWQMPCPMQPIRASADMFDC---   146
TabZIP98A     MDIQEECPPDDPLLRPWQTPYPATAMPIAATATHMLQY   157
              :** *     : : .*** * *   :  :*   .
```

C.

```
AtHSFB1/4     ---------------MTAVTAAQRSVPAPFLSKTYQLVDDHSTDDVVSWNEEGTAFVVWK      45
TaHSFB1A      MAGAAAQQQKGGSGGGGGAVRVGGGGPAPFLTKTHQMVEERGTDEVISWSEHGRSFVVWK      60
                          ... .  . *****:**:*:*:::.**:*:**.*.* :*****

AtHSFB1/4     TAEFAKDLLPQYFKHNNFSSFIRQLNTYGFRKTVPDKWEFANDYFRRGGEDLLTDIRRRK      105
TaHSFB1A      PVELARDLLPLHFKHCNFSSFVRQLNTYGFRKVVPDRWEFANENFRRGEQSLLSGIRRRK      120
               .*:*:**** :*** *****:**********.***:*****: **** :.**:.*****

AtHSFB1/4     SVIASTAGKCV------VV---------GSPSESNSGGGDDHGSSSTSSPGSSKNPGSVE      150
TaHSFB1A      ATGTTTPQSSKTCGTGVNVAFPPPLPALPPASASTSGTGNDHSTSSASSP----------      170
              :. ::*  ..        *            * *.** *:**.:**:***

AtHSFB1/4     NMVADLSGENEKLKRENNNLSSELAAAKKQRDELVTFLTGHLKVRPEQIDKMIKGGKFKP      210
TaHSFB1A      -TRPDLSSENEQLRKDNHALAAELALARRHCEELLGFLSRFLDVRQLDLRLLMDEDMQGA      229
                  ***.***:*:::*: *::*** *::: :**: **: .*.**  ::  ::. .

AtHSFB1/4     VESDEESECEGCDGGGGAEEGVGEGLKLFGVWLKGERKKRDRDEKNYVV--SGSRMTEIK      268
TaHSFB1A      A---------GGARSADQEHCCEKKVKLFGVVLKDASARKRGRCDEAAASERSMKMTR--      278
              .         *   ... *.   : :***** **.   ::    .: ..   . :**.

AtHSFB1/4     NVDFHAPLWKSSKVCN-------  284
TaHSFB1A      ---IGEPWVGVPSSCPARCGGGN  298
                 :  *     . *
```

D.

```
AtATL56      MPPTNNYRISGEPPSTTPSHPPPKPKTRILSLFLV-GVIMFSIFFLFLVLIGIASVLILP       59
TaATL56      ----------------------MARMAVSVLFLIAGVVLMLALHVLVIVWAVRRG----       33
                                  :  :  ***: **:::  :.:::::: .:

AtATL56      LLLSSLHRHHRRRRRNRRQESSDGLSSRFVKKLPQFKFSEPSTYTRYESDCVVCFDGFRQ       119
TaATL56      ---AVLRLRGAARERDQEQAEAAGLTADELGELPCQDFKAAAAVGTGAGECAVCLEAFQG       90
               : *: :   *.*::.* .: **::  : :**  .*.  ::     .:*.**::.*:

AtATL56      GQWCRNLPGCGHVFHRKCVDTWLLKASTCPICRARVRLWEEDPQEGELWRCFGHRRSSLL       179
TaATL56      GDRCRVLPGCHHGFHAQCVDSWLRQSRRCPVCRAEVACRGKAA-------------DAVV       137
             *: ** **** * ** :***:** ::  **:***.*    :              .:::

AtATL56      DL---------------- 181
TaATL56      DETATSEIVAERLGGADR 155
             *
```

E.

```
AtTCP20      MDPKNLNRHQVPNFLNPPPPPRNQGLVDDDAASAVVS-----------------------       37
TaTCP20      MD---------PKF--PPPPPLNKAEPTSAATATTTSSTSTPPAQQQHQQLDREQYRHPP       49
             **         *:*  ***** *:.   . *:::..*

AtTCP20      -DENRKPTTEIKDFQIVVSASD--------------------KEPNKKSQNQNQLGPKRS       76
TaTCP20      QQQEQQPPQHQQHLQIQVHQQQEDGGGGGGGGKEQQQQQVVVAAAAAGDRRVQALGPKRS       109
              ::::::  . :.:** *  .:                          .:. : ******

AtTCP20      SNKDRHTKVEGRGRRIRMPALCAARIFQLTRELGHKSDGETIQWLLQQAEPSIIAATGSG       136
TaTCP20      SNKDRHTKVDGRGRRIRMPALCAARIFQLTRELGHKSDGETVQWLLQQAEPAIVAATGTG       169
             *********:*********************************:*********:*:****:*

AtTCP20      TIPASALASSAATSNHHQGGSLTAGLMISHDLDGGSSSSGRPLNWGIGGG-EGVSRSSLP       195
TaTCP20      TIPASALSSVAPSLPSPTS-AL-AGRPH-----------HHHHMWGPPPASAGFSQA---       213
             *******:* * :     . :* **               :   **   .  *.*::

AtTCP20      TGLWPNVAGFGSGVPTTGLMSEGAGYRIGFP-GFDFPG------VGHMSFASILG-GNHN       247
TaTCP20      -GF-MNSSGA------DGGGIGGLMQRIGLPAGIELPGGGAGGMGGHIGFAPMFAGHAAA       265
              *:  * :*        *    *   ***:* *:::**       **:.** ::.

AtTCP20      QMPGLELGLSQEGNVGVLNPQSFTQIYQQMGQAQAQAQGRVLH---HMHHNHEE------       298
TaTCP20      AIPGLELGLSQEGHIGVLS-----QFYHQVGGAAGA-SGQLQHPHPHQHHHHEQHHHQQQ       319
              :***********::***.     *:*:*:* * .  .*:: *   * **:**:

AtTCP20      -HQQESGEKDDSQGSGR-----     314
TaTCP20      QQQQEDGEDEREDGDSEEESGQ     341
              :***.**.: .:*...
```

F.

```
AtPHL6      MYIKAIMNRHRLLSAATDECNKKLGQACSSSLSPVHNFLNVQPEHRKTPFIRSQSPDSPG        60
TaPHL6      ------MSTQNVIPMKHIIAPDIRAHTCNAPQPSVHQMFNAKSDIY------SS-ADDTS        47
                  *. :.::      . .  .::*.:    **:::*.: :        *.  *. .

AtPHL6      QLWPKNSSQSTFSRSSTFCTNLYLSSSSTSETQKHLGNSLPFLPDPSSYTHSAS-GVESA        119
TaPHL6      RVSYADLSDPNSSSSSTFCTSMYSSSSTKP-------SGFSFLPHPSKCEQQQVSAAKSL        100
            ::   : *: . * ******.:* ***:.        ..: ***.**.  :.   ..:*

AtPHL6      RSPSIFTEDLGNQCDGGNS-GSLLKDFLNLSGDACSDGDFHDFGCSNDSYCLSDQMELQF        178
TaPHL6      SSSLLFAADLSTGVHGDLEHPLDLKDFLNLSGNASDS----SFRAGGNAMDFSEQLEFQF        156
             *  :*: **..  .*. .    *********:*...    .* ...::  :*:*:*:**

AtPHL6      LSDELELAITDRAETPRLDEIYETPLASNPVTRLSPSQ--SCVPG--AMSVDVVSSHPSP        234
TaPHL6      LSEQLGIAITDNEEIPRLDDIYGIPPQCSPILVSPSSDHEGLRSGGSPVKVQLSSSPSSS        216
            **::* :****. * ****:**  *  ..*:     *:  .   *   :.*:: **  *

AtPHL6      GSAANQKSRMRWTPELHESFVKAVIKLEGPEKATPKAVKKLMNVEGLTIYHVKSHLQKYR        294
TaPHL6      GATACSKTRMRWTLELHERFVEALKKLGGPEKATPKGVLKLMKVEGLTIFHVKSHLQNYR        276
            *::* .*:***** **** **:*: ** ********.* ***:******:*******:**

AtPHL6      LAKYMPEKKEEKRTDNSEEKKLALSKSEADEKKKGAIQLTEALRMQMEVQKQLHEQLEVQ        354
TaPHL6      HVKYIPEKKEVKRTCSEDNKA--KSAPGIDSGKKKSFQMAEALRMQMEVQKQLHEQLEVQ        334
             .**:***** *** ..::*    *    *. ** ::*::********************

AtPHL6      RVLQLRIEEHAKYLEKMLEEQRKTGRWISSSSQTVLSPSDDSIPDSQNMSKTKASSPQPP        414
TaPHL6      RKLQLRIEEHARYLQQILEQQKARKSPVPKPKEE-------TEVN-----TT----SAPS        378
            * *********:**:::**:*:     : . .:        :  :     .*      *

AtPHL6      LPAENKASETEDDKC-ESPQKRRRLENIAESEDPKR        449
TaPHL6      LKR--KLSDTKIEHNSQMDSRRPELQ-LDLESEP--        409
            *    * *:*: ::  :  .:* .*: :  ..:*
```

Figure 4.7: Protein alignments of Arabidopsis Y1H targets with wheat homologues. Alignments are between the Arabidopsis targets and their closest wheat homologues, which were identified through database searches. The alignments were made using Clustal Omega[195,196]. A. AtbZIP1 B. AtbZIP53 C. AtHSFB1/4 D. AtATL56 E. AtTCP20 F. AtPHL6. Sequences from the A genome were chosen for the wheat homologues.

Arabidopsis gene	Wheat homologue	Identity score	Expect value
AtbZIP1	TabZIP2	52%	2e-17
AtbZIP53	TabZIP98	45%	1e-38
AtHSFB1/4	TaHSFB1	43%	1e-63
AtATL56	TaATL56	32%	6e-22
AtTCP20	TaTCP20	51%	3e-53
AtPHL6	TaPHL6	52%	1e-89

Table 4.1: Identity score and expect values for Y1H targets wheat homologues. Identity scores and expect values of the Arabidopsis and wheat homologues were determined aligning the protein sequences using NCBI blast tool[150].

4.5 TabZIP2 binds to TaWRKY19 promoter

Once the wheat homologues had been identified I had to show that the wheat homologues also bound to TaWRKY19 promoter. The targets were narrowed down based on their publications in Arabidopsis, which left me with four targets to experiment with, TabZIP2, TabZIP98, TaTCP20 and TaHSFB1. Primers were designed for the four genes amplifying the CDS of each gene, starting from the ATG start codon until the stop codon. After multiple attempts, with differing conditions the CDS for TabZIP2 was cloned (figure 4.8) using cDNA from Septoria infected wheat samples (12dpi). It has a DNA sequence length of 453bp, which corresponds to the size of the band in the figure.

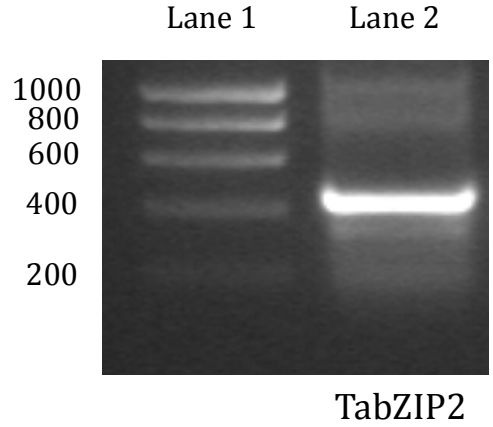

Figure 4.8: Gel of cloning PCR for TabZIP2 CDS. PCR products were run on a 1% agarose gel in 1xTAE buffer for size separation and visualised under UV light with quantity one software. A 1kb hyperladder was used for size indication (lane 1). The expected size for TabZIP2 (lane 2) was 453bp.

Following the same method as previously mentioned the DNA was cloned into pDEST22 to be tested for binding to TaWKRY19 promoter through a Y1H experiment. Firstly the two yeast strains were mated and then screened for

mating progeny by growing the cultures on minimal SD base media containing amino acid mix –L-W. Colonies from these plates were then grown up in liquid media and pipetted onto the selection plates. As can be seen in figure 4.9 TabZIP does bind to TaWRKY19 promoter, if slightly weakly, up to 60mM of 3AT. This binding is stronger than the empty vector pDEST22 binding on TaWRKY19 promoter.

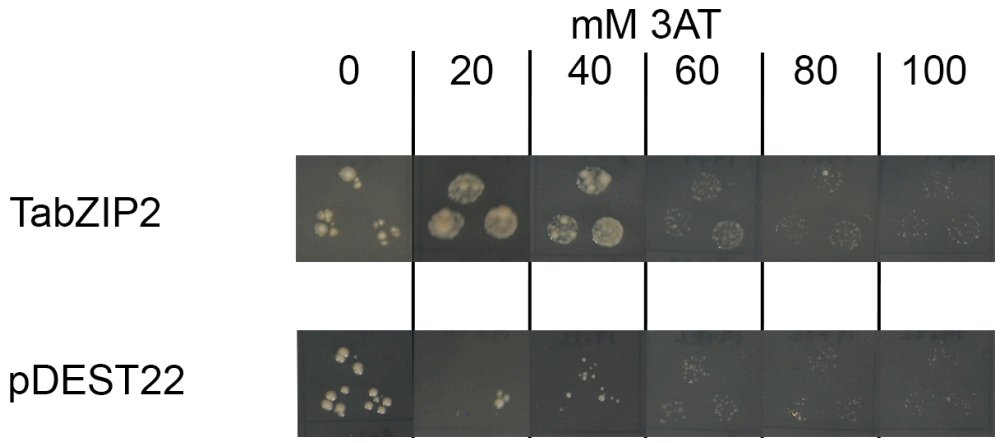

Figure 4.9: Y1H experiment for TabZIP2 TF and TaWRKY19 promoter. TabZIP2 TF and pDEST22 (negative control) were mated with TaWRKY19 promoter and grown on increasing minimal SD base with amino mix –L-W-H and 3AT selection (20mM, 40mM, 60mM, 80mM and 100mM). Colonies were grown at 28^0C for 3 days before pictures were taken.

4.6 TabZIP2

The original aim of the Y1H experiment was to find TFs that potentially had a greater influence in defence against Septoria. Since the wheat homologue of AtbZIP1 also bound to the TaWRKY19 promoter, we were hopeful that it would have a role in defence.

To determine a possible role, the structure of TabZIP2 was investigated. It is a small protein of 16.3kDa, which belongs to the S1 family, along with AtbZIP1. This family is generally small, containing just the bZIP domain, with an extended leucine zipper region. The bZIP domain is highlighted in red on the protein sequence (figure 4.10).

A.

MSSPSRRSSSPESNIDGGSGSGSAGDERKRKRMLSNRESARRSRARKQQRMEELIA
EASRLQAENKRVEAQIGAYTTELTKVDGENAVLRARHGELAGRLQALGGVLEIFQ
VAGAPVDIPEIPDPLLRPWQSPFAPQLATAGGMPDAFQF

B.

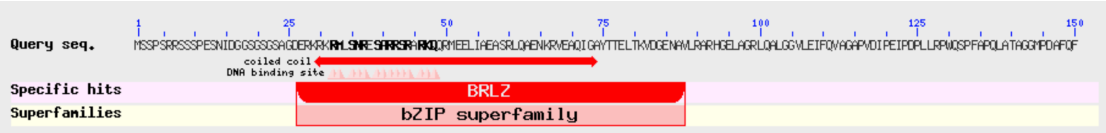

Figure 4.10: Protein sequence of TabZIP2. A. Protein sequence of TabZIP2 (150aa). Highlighted in red is the bZIP domain. B. Cartoon representation showing the position of the bZIP domains relative to the protein. This was generated using NCBI protein blast tool[150].

As with TaWRKY19, testing the expression of TabZIP2 over a time course of healthy and Septoria infected tissue can give an insight into a potential link to defence. The same three independent replicates of the time courses were used in this experiment. qRT-PCR primers for TabZIP2 were designed and tested to ensure they only bound to TabZIP2 and tested for the appropriate efficiency levels in qRT-PCR. Once a suitable primer pair was designed, I ran the qRT-PCR using duplicate wells. The results can be seen in figure 4.11. There is higher variation in TabZIP2 expression than TaWRKY19 expression in the healthy samples. After Septoria infection the expression increases above the expression level in the healthy samples. This occurs between 10-14dpi, with the highest peak being at 14dpi. Like TaWRKY19 expression, this change coincides with the switch from biotrophic to necrotrophic growth at 12dpi. At the highest point (10dpi) the expression is five fold higher than in the healthy samples of the same time point. Compared to TaWRKY19, TabZIP2's expression increases later (10dpi) post infection, and decreases earlier (16dpi).

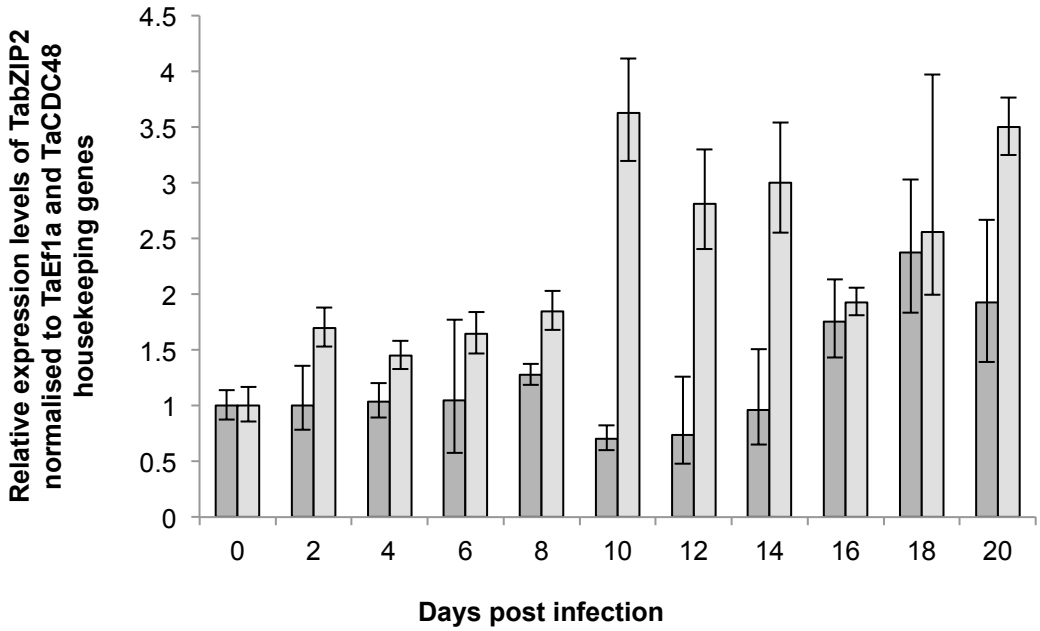

Figure 4.11: TabZIP2 expression in healthy and Septoria infected time course. RNA samples were collected every 2 days from healthy (light grey) and Septoria infected (dark grey) seedlings of 4-weeks-old. Leaves from three separate plants were collected per sample. qRT-PCR was performed using TaCDC48 and TaEF1a as housekeeping genes. This experiment was repeated 3 times. Two replicate wells for each sample were performed in the qRT-PCR. Error bars represent +/- 1 standard error.

4.7 TaBZIP2 silencing using VIGS

Since TabZIP2 also showed a difference in expression pattern after Septoria infection, we (Ari Sadanandom and myself) decided to further investigate its role. To do this two silencing fragments were designed against TabZIP2 gene. Unlike TaWRKY19, the two fragments were designed to bind to the CDS region of the gene. The fragments are 205bp and 214bp long, they can be seen in figure 4.12, which shows the CDS sequence with the two silencing fragments highlighted in blue.

atgtcgtcgccgtcgcgccggagctccagccccgagagcaacatcgacggcggcagcggcagcggctccgcc
ggtgacgagcgcaagcgcaagaggatgctgtccaacagggagtcggcgaggcggtcccgcgctcgcaagca
gcagcggatggaggagctcatcgccgaggccagccgcctccaggccgagaacaagcgcgtggaggcccaga
tcggcgcctacacgaccgagctgaccaaggtggacggcgagaacgccgtgctccgcgcgcgccacggcgag
ctcgccggccggctgcaggcgctcggcggcgtcctggagatcttccaggtggccggcgcgcccgtggacatcc
cggagatccctgacccgctgctccgcccatggcagtccccgttcgcgccccagctggccaccgccggcggcat
gcctgacgcgttccagttc**tga**

Figure 4.12: DNA sequence of CDS of TabZIP2. TabZIP2 CDS (453bp) with ATG start codon and TGA stop codon highlighted in bold. Silencing fragments TabZIPA and TabZIP2B are highlighted in blue

Unfortunately, at the time of performing this experiment, I did not have access to KWS database to test the silencing fragments for efficiency. However, due to their size (over double that of TaWRKY19A and TaWRKY19B fragments), there was less of a concern about testing their silencing efficiency compared to TaWRKY19 silencing fragments.
A blast search[151] was performed for each silencing fragment against the most up-to-date wheat genome assembly (figure 4.13). For TabZIP2A, there is some potential off target silencing, as there is some homology to TabZIP98. TabZIP2B is more specific; potentially showing some minor off target silencing against two regions of the genome.

A.

TabZIP2 chromosome 1AS

```
GAGAGCAACATCGACGGCGGCAGCGGCAGCGGCTCCGCCGGTGACGAGCGCAAGCGCAAGAGGATGCTGTCCAACAGGGAGTCGGCGAGGCGGTCCCGCG
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GAGAGCAACATCGACGGCGGCAGCGGCAGCGGCTCCGCCGGTGACGAGCGCAAGCGCAAGAGGATGCTGTCCAACAGGGAGTCGGCGAGGCGGTCCCGCG
CTCGCAAGCAGCAGCGGATGGAGGAGCTCATCGCCGAGGCCAGCCGCCTCCAGGCCGAGAACAAGCGCGTGGAGGCCCAGATCGGCGCCTACACGACCGA
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CTCGCAAGCAGCAGCGGATGGAGGAGCTCATCGCCGAGGCCAGCCGCCTCCAGGCCGAGAACAAGCGCGTGGAGGCCCAGATCGGCGCCTACACGACCGA
GCTGA
|||||
GCTGA
```

TabZIP2 chromosome 1DS

```
GAGAGCAACATCGACGGCGGCAGCGGCAGCGGCTCCGCCGGTGACGAGCGCAAGCGCAAGAGGATGCTGTCCAACAGGGAGTCGGCGAGGCGGTCCCGCG
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GAGAGCAACATCGACGGCGGCAGCGGCAGCGGCTCCGCCGGTGACGAGCGCAAGCGCAAGAGGATGCTGTCCAACAGGGAGTCGGCGAGGCGGTCCCGCG
CTCGCAAGCAGCAGCGGATGGAGGAGCTCATCGCCGAGGCCAGCCGCCTCCAGGCCGAGAACAAGCGCGTGGAGGCCCAGATCGGCGCCTACACGACCGA
| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||  ||||||||||||||||||||||||||||||||||||||
CGCGCAAGCAGCAGCGGATGGAGGAGCTCATCGCCGAGGCCAGCCGCCTCCAGGCCGAGAACGCGCGCGTGGAGGCCCAGATCGGCGCCTACACGACCGA
GCTGA
|||||
GCTGA
```

TabZIP2 chromosome 1BS

```
GAGAGCAACATCGACGGCGGCAGCGGCAGCGGCTCCGCCGGTGACGAGCGCAAGCGCAAGAGGATGCTGTCCAACAGGGAGTCGGCGAGGCGGTCCCGCG
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GAGAGCAACATCGACGGCGGCAGCGGCAGCGGCTCCGCCGGTGACGAGCGCAAGCGCAAGAGGATGCTGTCCAACAGGGAGTCGGCGAGGCGGTCCCGCG
CTCGCAAGCAGCAGCGGATGGAGGAGCTCATCGCCGAGGCCAGCCGCCTCCAGGCCGAGAACAAGCGCGTGGAGGCCCAGATCGGCGCCTACACGACCGA
| ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||  ||||||||||||||||||||||||||||||||||||||
CGCGCAAGCAGCAGCGGATGGAGGAGCTCATCGCCGAGGCCAGCCGCCTCCAGGCCGAGAACGCGCGCGTGGAGGCCCAGATCGGCGCCTACACGACCGA
GCTGA
|||||
GCTGA
```

TabZIP98 chromosome 5DL (off target)

```
GACGAGCGCAAGCGCAAGAGGATGCTGTCCAACAGGGAGTCGGCGAGGCGGTCCCGCGCTCGCAAGCAGCAGC
|| ||||| | |||   ||||||| |||||||| | |||||||||||||||||| |||   |||||||||| ||
GAGGAGCGGAGGCGGCGGAGGATGGTGTCCAACCGCGAGTCGGCGAGGCGGTCGCGCATGCGCAAGCAGCGGC
```

Predicted protein phosphatase 2C (off target)

```
GACGAGCGCAAGCGCAAGAGGATGCTGTCCAACAGGGAGTCGGCGAGGCGGTCCCGCGCTCGCAAGCAGCAGC
|| ||||| | |||   ||||||| |||||||| | |||||||||||||||||| |||   |||||||||| ||
GAGGAGCGGAGGCGGCGGAGGATGGTGTCCAACCGCGAGTCGGCGAGGCGGTCGCGCATGCGCAAGCAGCGGC
```

B.

TabZIP2 chromosome 1AS

```
CAAGGTGGACGGCGAGAACGCCGTGCTCCGCGCGCGCCACGGCGAGCTCGCCGGCCGGCTGCAGGCGCTCGGCGGCGTCCTGGAGATCTTCCAGGTGGCC
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CAAGGTGGACGGCGAGAACGCCGTGCTCCGCGCGCGCCACGGCGAGCTCGCCGGCCGGCTGCAGGCGCTCGGCGGCGTCCTGGAGATCTTCCAGGTGGCC
GGCGCGCCCCGTGGACATCCCGGAGATCCCTGACCCGCTGCTCCGCCCATGGCAGTCCCCGTTCGCGCCCCAGCTGGCCACCGCCGGCGGCATGCCTGACG
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GGCGCGCCCGTGGACATCCCGGAGATCCCTGACCCGCTGCTCCGCCCATGGCAGTCCCCGTTCGCGCCCCAGCTGGCCACCGCCGGCGGCATGCCTGACG
CGTTCCAGTTCTGA
|||||||||||||||
CGTTCCAGTTCTGA
```

TabZIP2 chromosome 1DS

```
CAAGGTGGACGGCGAGAACGCCGTGCTCCGCGCGCGCCACGGCGAGCTCGCCGGCCGGCTGCAGGCGCTCGGCGGCGTCCTGGAGATCTTCCAGGTGGCC
|||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
CAAGGTGGACGGCGAGAACGCCGTGCTCCGCGCGCGCCACGGCGAGCTCGCCGGGCGGCTGCAGGCGCTCGGCGGCGTCCTGGAGATCTTCCAGGTGGCC
GGCGCGCCCCGTGGACATCCCGGAGATCCCTGACCCGCTGCTCCGCCCATGGCAGTCCCCGTTCGCGCCCCAGCTGGCCACCGCCGGCGGCATGCCTGACG
||||| |||||||||||||||||||||||| |||||||||||||||| |||||||||||||||||||||||||||||||| ||||||| |||||||| ||||
GGCGCTCCCGTGGACATCCCGGAGATCCCCGACCCGCTGCTCCGCCCGTGGCAGTCCCCGTTCGCGCCCCAGCTGGCCGCCGCCGGTGGCATGCCCGACG
CGTTCCAGTTCTGA
|||||||||||||||
CGTTCCAGTTCTGA
```

TabZIP2 chromosome 1BS

```
CAAGGTGGACGGCGAGAACGCCGTGCTCCGCGCGCGCCACGGCGAGCTCGCCGGCCGGCTGCAGGCGCTCGGCGGCGTCCTGGAGATCTTCCAGGTGGCC
|||||| |||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||||| ||||||
CAAGGTTGACGGCGAGAACGCCGTGCTCCGCGCGCGCCACGGCGAGCTCGCCGGGCGGCTGCAGGCGCTCGGCGGCGTCCTGGAGATCTTCCATGTGGCC
GGCGCGCCCGTGGACATCCCGGAGATCCCTGACCCGCTGCTCCGCCCATGGCAGTCCCCGTTCGCGCCCCAGCTGGCCACCGCCGGCGGCATGCCTGACG
|| |||||||| |||||||| |||||||| |||||||||||||||||||||||||||||||| ||||||||||||||| ||||||| |||||||| ||||
GGTGCGCCCGTTGACATCCCAGAGATCCCGGACCCGCTGCTCCGCCCATGGCAGTCCCCGTTTGCGCCCCAGCTGGCCGCCGCCGGTGGCATGCCGGACG
CGTTCCAGTTCTGA
|||||||||||||||
CGTTCCAGTTCTGA
```

Gene of unknown function unscaffolded (off target)

```
GCTCGGCGGCGTCCTGGAGATCTTCCAGGTGGC
||||||||||||||||||||||||||  ||||||
GCTCGGCGGCGTCCTGGAGATCTTC--GGTGGC
```

Gene of unknown function chromosome 2DS (off target)

```
GCTCGGCGGCGTCCTGGAGATCTTC
||||||||||||||||||||||||
GCTCGGCGGCGTCCTGGAGATCTTC
```

Figure 4.13: Blast searches of TabZIP2 silencing fragments. The Earlham Institute's[151] blast function was used to test off target silencing of A. TabZIP2A and B. TabZIP2B silencing fragments. Parameters were set to identify homology of sequences over 16bps long, against the cv. Chinese Spring wheat genome sequence. This is based on the size of siRNA produced by DICER cleavage[104,105].

Primers were designed to clone each of the silencing fragments. A PCR reaction was performed (55^{0}C annealing temperature, 30 cycles and 10 seconds extension time), using Septoria infected cDNA(12dpi) and Q5 DNA polymerase to amplify the silencing fragments. The PCR products were the run a 1% agarose gel for separation, this was then visualised using UV light. This can be seen in figure 4.14. As described in chapter 3.4, these fragments were then cloned into BSMVγ RNA containing vector and eventually transformed into *A. tumefaciens*, ready for infiltration into *N. benthamiana* and silencing.

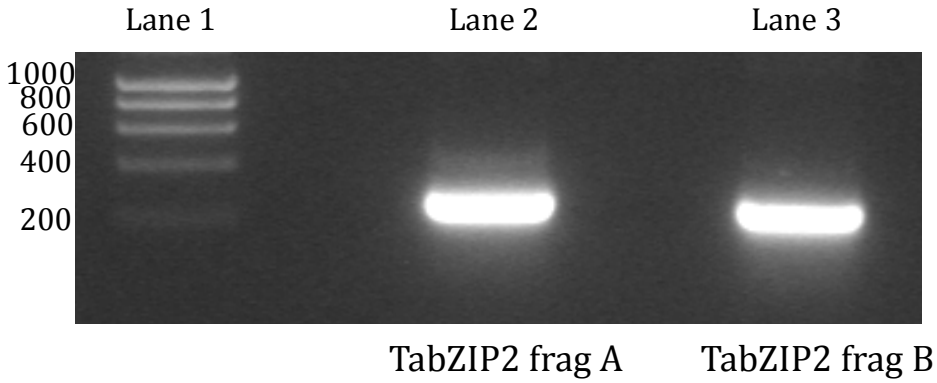

Figure 4.14: Gel of cloning PCR for TabZIP2A and TabZIP2B silencing fragments. PCR products were run on a 1% agarose gel in 1xTAE buffer for size separation and visualised using UV light with quantity one software. A 1kb hyperladder was used for size indication (lane 1). The expected size for TabZIP2A (lane 2) and TabZIP2B (lane 3) was 205bp and 214bp respectively.

Two-week-old wheat seedlings were then silenced with both of these fragments, BSMV:PDS and BSMV:00. Two weeks after silencing treatment, and after the development of PDS photobleaching phenotype in BSMV:PDS treated plants, leaf tissue samples were collected. The RNA was extracted and cDNA synthesised ready for qRT-PCR to check the levels of TabZIP expression and therefore silencing. Three independent repeats were performed, pooling three leaf samples each time, with two well replicates in each qRT-PCR. Figure 4.15 shows the results of this experiment. Silencing

levels were similar for both silencing fragments. Wheat silenced with BSMV:TabZIP2A had 40% less expression than BSMV:00 (on average) and wheat silenced with BSMV:TabZIP2B had 41% less expression than BSMV:00 (on average). Unfortunately the differences are not significant, with p values of 0.15 and 0.16 for BSMV:TabZIP2A and TabZIP2B respectively.

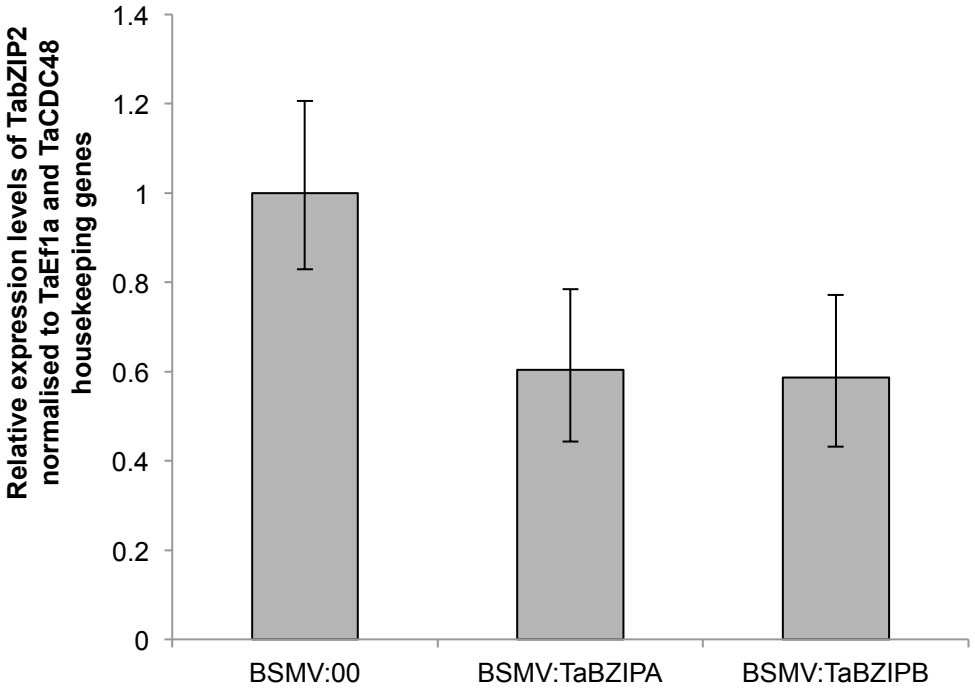

Figure 4.15: qRT-PCR to show silencing of TabZIP2 in wheat. Fold change of TabZIP2 in wheat silenced with BSMV:TabZIP2A and BSMV:TabZIP2B compared to BSMV:00 control. RNA was extracted 2 weeks post silencing treatment. Leaves from three separate plants were collected per sample. qRT-PCR was performed using TaCDC48 and TaEF1a as housekeeping genes. This experiment was repeated 3 times. Two replicate wells for each sample were performed in the qRT-PCR. Error bars represent +/- 1 standard error.

4.8 TabZIP2 in wheat defence against Septoria

After confirming TabZIP2 could be silenced using the two silencing fragments, the silenced wheat (4-weeks-old) was used to test whether Septoria's infection changes when compared to the control silenced wheat.

Figure 4.16 shows the infection symptoms that developed from 11dpi to 21dpi. Pictures were taken everyday between 2-3pm with the pictures in figure 4.16 showing an average symptom development across the 3 experiments. Symptoms for wheat silenced with TabZIP2 have later onset of Septoria symptoms, beginning at 13dpi, whereas symptoms on BSMV:00 treated wheat begin at 12dpi. Symptoms develop at the same rate for both the control and TabZIP2 silenced wheat, taking 9 days from onset of infection symptoms until complete leaf death (20dpi for BSMV:00 and 21dpi for BSMV:TabZIP2A and BSMV:TabZIP2B).

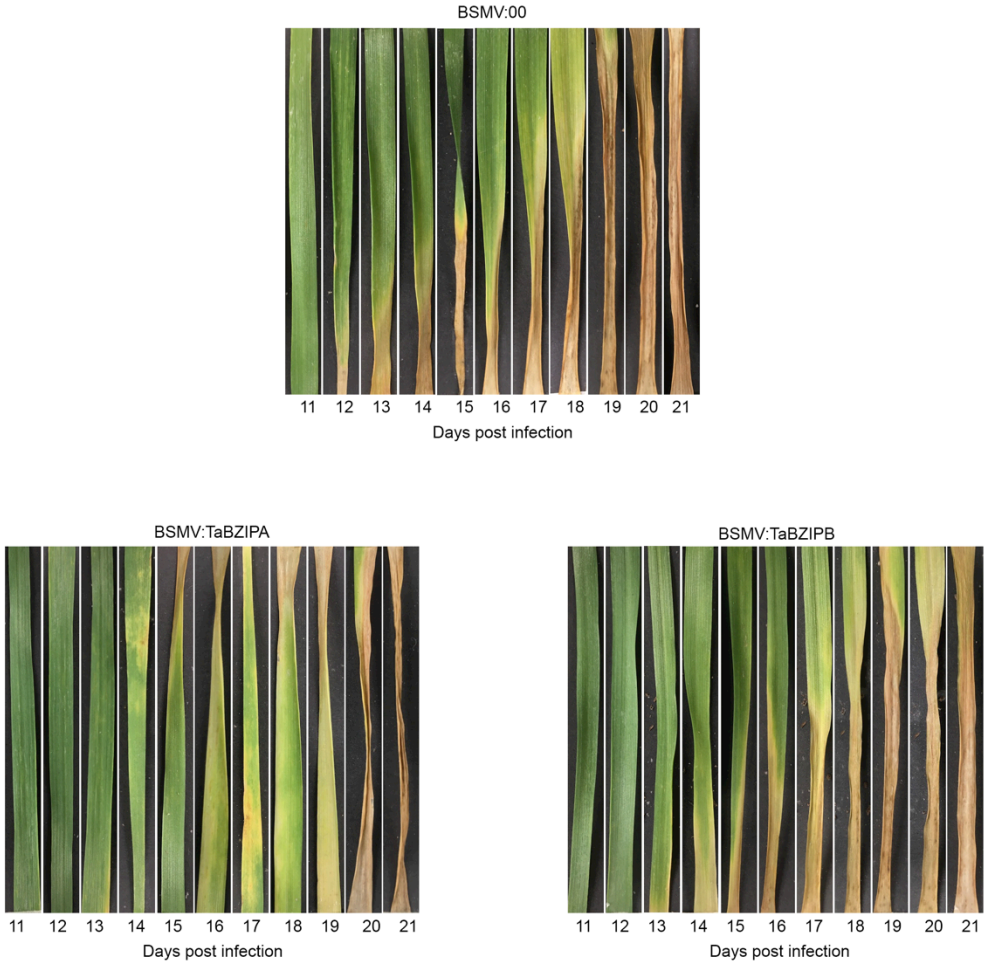

Figure 4.16: Septoria infection symptoms on TabZIP2 silenced wheat. Four-week-old-seedlings, which had undergone silencing treatment (BSMV:00, BSMV:TabZIP2A and BSMV:bZIP2B) were stuck down onto black card and infected with Septoria (abaxial and adaxial). The seedlings were grown under high humidity conditions to encourage Septoria infection. The infection was then followed daily, with photos taken between 2-3pm. In this figure the initial stages of the infection symptoms are shown, from 11dpi until 21 dpi. Photos are representative of the symptoms seen in 3 independent experiments.

After the infection cycle was complete the pycnidia were counted on each leaf over a 2cm leaf length. The results of this are shown in figure 4.17. BSMV:TabZIP2B silenced wheat have, on average, fewer pycnidia than BSMV:00, however this number is not statistically different. BSMV:TabZIP2A silenced wheat has, on average, slightly more pycnidia, but again this number is not statistically significant. Therefore it should be said that there is no difference in the amount of pycnidia in wheat silenced with TabZIP2.

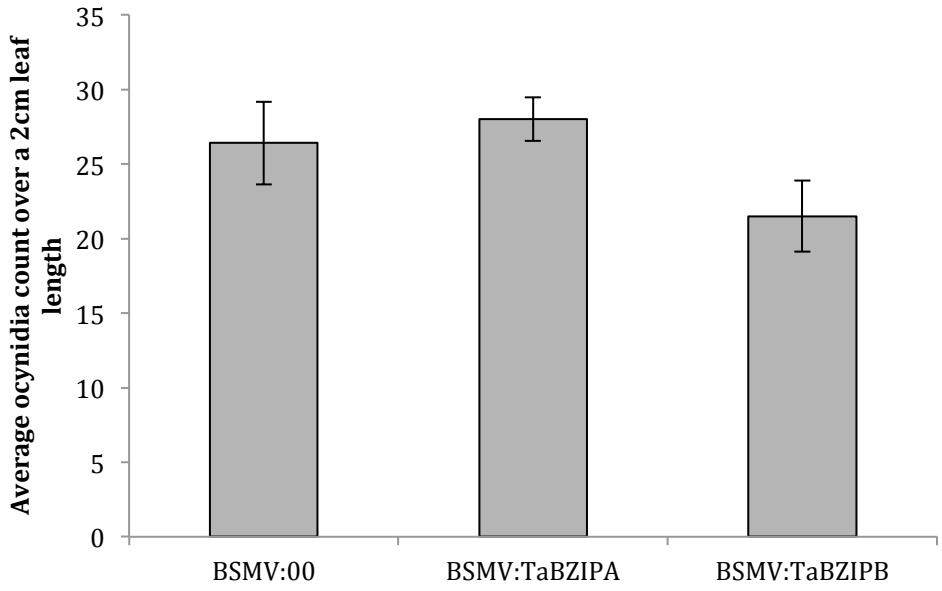

Figure 4.17: Pycnidia count from Septoria infected, TabZIP2 silenced wheat leaves. Pycnidia were counted over a 2cm leaf length per leaf. Error bars correspond to +/- 1 standard error. The experiment was repeated independently 3 times with 5 leaves per experiment counted.

After counting the pycnidia, the spores were also counted. The results of this can be seen in figure 4.18. Although the pycnidia count showed little difference between the control and TabZIP2 silenced wheat, this is not the same for the spore count. BSMV:TabZIP2A and BSMV:TabZIP2B silenced wheat both have a reduction in Septoria sporulation with 23% and 33% less spores respectively when compared to BSMV:00. Both of these differences are statistically significant with over 99% confidence (1.12E-03 and 1.75E-05 for BSMV:TabZIP2A and BSMV:TabZIP2B respectively).

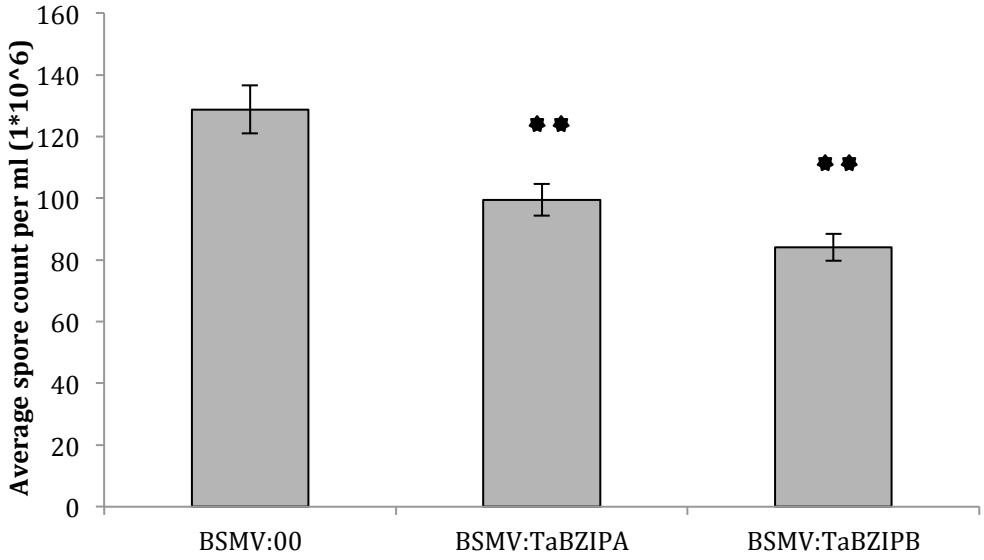

Figure 4.18: Septoria spore counts from TabZIP2 silenced and infected leaves. Five leaves were submerged in 10ml of water and vortexed to wash the spores from the pycnidia. Spores were then counted using a haemocytometer under a light microscope (x10 magnification). Four spores counts across independent 4x4 squares on the haemocytometer were performed per spore suspension. Error bars represent +/- 1 standard error. This experiment was repeated independently 3 times. Double asterisks are used to denote a results difference from the control of $p<0.01$ respectively.

Combining the effects silencing TabZIP2 has upon the infection symptom progression and spore count, it can be said that TabZIP2 does have a role in wheat defence against Septoria. Potentially by reducing the capacity of the pycnidia to produce spores.

4.9 Investigating TabZIP2s regulation of TaWRKY19

According to the Y1H performed (figure 4.9), TabZIP2 binds to TaWRKY19 promoter, potentially regulating its expression. It was decided to investigate this further, with the aim of finding out whether TabZIP2 was a positive or negative regulator of TaWRKY19 expression.

Initial investigations were based on looking at different varieties of wheat and seeing if there was any correlation between resistance to Septoria,

TaWRKY19 expression and TabZIP2 expression. Seven different winter wheat varieties were chosen. Septoria resistance is defined in a 1-9 scale, with one having no resistance and nine high resistance. In the current recommended list the highest resistance level is 7.4 (LG Sundance), with no other varieties over 7 on the list[13]. The seven varieties I have chosen (out of those available to me at the time) cover the lower end of the scale (Santiago, 4) and increase towards the higher levels of resistance (Gator and Lili, 6) as shown in figure 4.19. More specific numbers were hard to find for each of the varieties therefore they were each rounded to the nearest whole number.

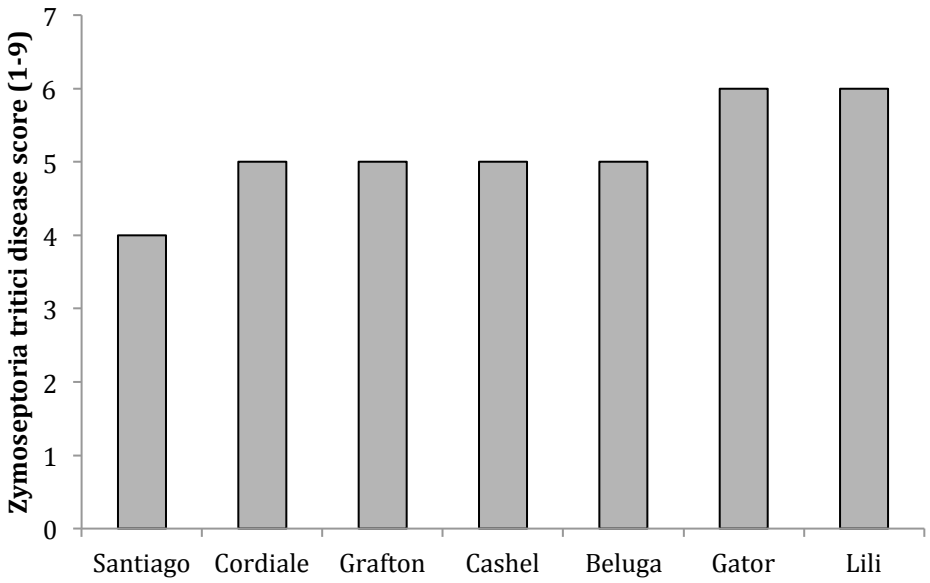

Figure 4.19: Septoria disease score for 7 winter wheat varieties. Scores were obtained from ADHB winter wheat recommended list[13].

Three seedlings of each variety were grown until they were 4-week-old, at which point leaf samples were collected from each variety (three leaves from three different seedlings per sample) for RNA extraction and cDNA synthesis. This was repeated, independently 3 times. Once these samples had been processed qRT-PCR was performed to measure the expression of TaWRKY19 and TabZIP2 in the different varieties. Each well was repeated twice and the housekeeping genes TaCDC48 and TaEF1a were used.

The results comparing TaWRKY19 expression are shown in figure 4.20. Across 5 of the varieties there appears to be a correlation between expression and resistance, with the more resistant wheat having, on average, higher levels of TaWRKY19 expression. However Beluga and Lili break this trend, with Beluga having the same expression level as Gator, which is 1 point higher in the resistance scale. Lili has the lowest expression of TaWRKY19 but has one of the highest resistant scores. There is little variation in TaWRKY19 expression across the varieties. Between the highest and the lowest there is only is a doubling in expression, with most of the varieties having a similar expression level.

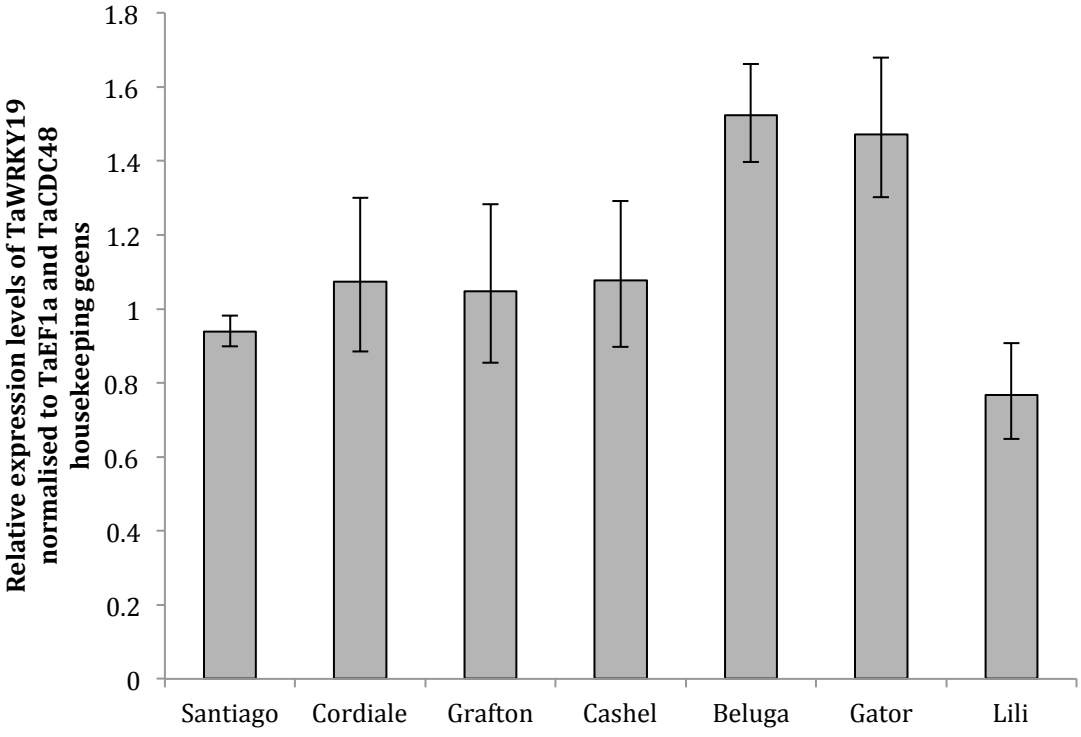

Figure 4.20: qRT-PCR of TaWRKY19 expression in 7 different winter wheat varieties. RNA was extracted from 4-week-old seedlings. Leaves from three separate plants were collected per sample. qRT-PCR was performed using TaCDC48 and TaEF1a as housekeeping genes. This experiment was repeated 3 times. Two replicate wells for each sample were performed in the qRT-PCR. Error bars represent +/- 1 standard error.

TabZIP2 expression (figure 4.21) is much more varied across the varieties; with 3 fold difference between the highest and the lowest (Grafton and Santiago respectively). There appears to be no correlation between the resistance scores for the varieties and TabZIP2s expression levels. Santiago does have the lowest expression level and the lowest resistance score, but after that there is little correlation with varieties at score 5 all having higher expression than the two varieties with a score of 6.

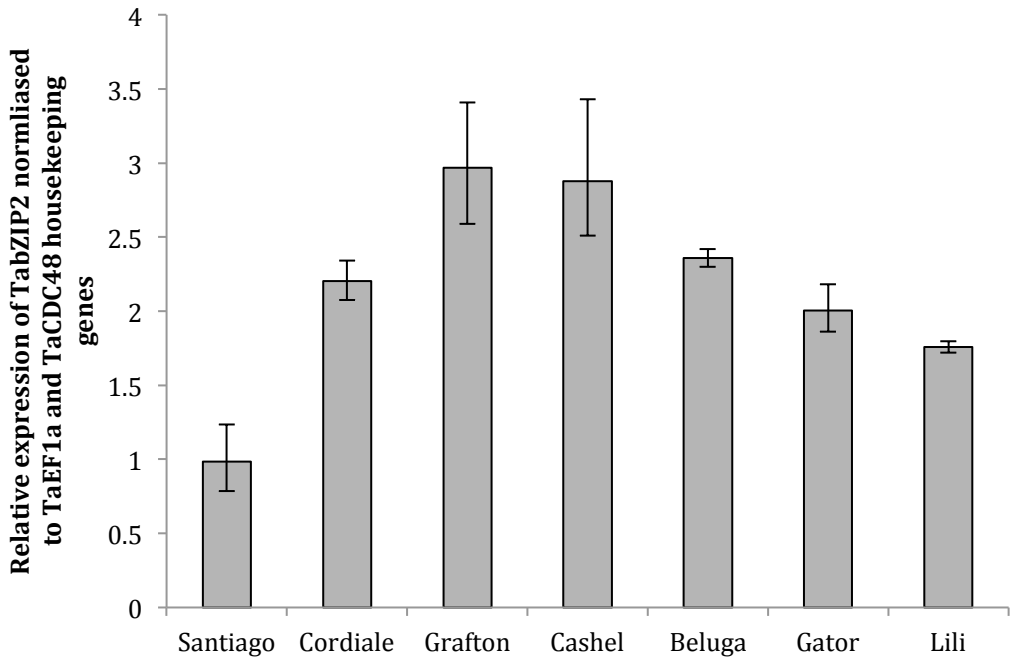

Figure 4.21: qRT-PCR of TabZIP2 expression in 7 different winter wheat varieties. RNA was extracted from 4-week-old seedlings. Leaves from three separate plants were collected per sample. qRT-PCR was performed using TaCDC48 and TaEF1a as housekeeping genes. This experiment was repeated 3 times. Two replicate wells for each sample were performed in the qRT-PCR. Error bars represent +/- 1 standard error.

As well as looking into the correlation between the varieties resistance score and the expression levels of TaWRKY19 and TabZIP2, the plan was to investigate any potential correlation between TaWRKY19 and TabZIP2. Unfortunately there appears to be no correlation between the two TFs. Grafton and Cashel, which have the highest levels of TabZIP2, have

expression levels in the middle of the range compared to the other varieties and vice versa with the varieties expressing TaWRKY19 the highest having a medium expression level of TabZIP2.
To further investigate whether there is a correlation, wheat with higher and lower scores should also be investigated and tested at older growth stages. The scores are determined in the field on adult plants so, in the future the use of older plants would have allowed a more field relevant scenario, however my time scale and growth space did not allow this to be carried out. Alternatively independent verification of the Septoria resistance scores through the seedling infection assays previously performed could also be used.

Next the TabZIP2 silenced wheat samples were used to test TaWRKY19 expression. This can be seen in figure 4.22. Wheat silenced with BSMV:TabZIP2A has, on average higher levels, of TaWRKY19 (1.45 times higher) but is not statistically significant (p value = 0.11) with the error bars almost crossing with the BSMV:00 control. BSMV:TabZIP2B treated wheat has a significantly higher expression of TaWRKY19 (p value = 0.022), with a fold change of 1.96 (on average) compared to the BSMV:00 control. As seen in figure 4.16, BSMV:TabZIPA and BSMV:TabZIPB treated wheat samples have very similar levels of TabZIP2 silencing (40% and 41% respectively), so I would have expected similar levels of TaWRKY19 in both of these samples. This experiment indicates that TabZIP2 is a negative regulator of TaWRKY19 expression, either by direct binding (as the Y1H results show) or indirectly through an unknown TF.

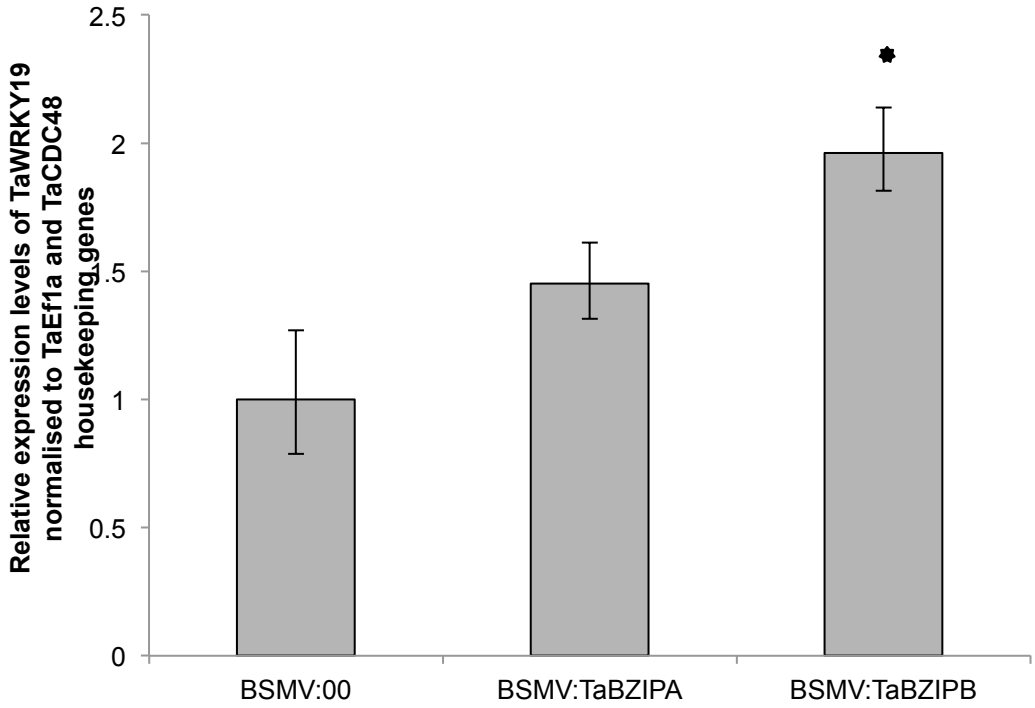

Figure 4.22: TaWRKY19 expression in TabZIP2 silenced wheat. qRT-PCR to measure TaWRKY19 expression in TabZIP2A and TabZIP2B silenced wheat compared to BSMV:00 control. RNA was extracted two weeks post silencing treatment (4-week-old seedlings). Leaves from three separate plants were collected per sample. qRT-PCR was performed using TaCDC48 and TaEF1a as housekeeping genes. This experiment was repeated 3 times. Two replicate wells for each sample were performed in the qRT-PCR. Error bars represent +/- 1 standard error. Asterisks are used to denote a results difference from the control of $p<0.05$.

As well as looking into the expression of TaWRKY19, the potential for TabZIP2 to regulate other TaWRKYs genes was also investigated. The expression levels of TaWRKY2, TaWRKY9, TaWRKY41 and TaWRKY63 were measured. However results for TaWRKY2 and TaWRKY9 were not conclusive so they are not included in this thesis. TaWRKY41 and TaWRKY63 are WRKY proteins that also have NBS-LRR domains before the WRKY domain. These proteins are important in the detection of pathogen effectors hence why I chose to study them. So far they are the only two NBS-LRR-WRKY proteins identified in wheat. qRT-PCR primers were designed for both of these genes

and tested for their ability to only amplify TaWRKY41 and TaWRKY63 and to ensure they were adequately efficient. These were then used to measure the expression levels of TaWRKY41 (figure 4.23) and TaWRKY63 (figure 4.24) in the TabZIP2 silenced wheat lines (3 repeats).

On average TaWRKY41 has slightly reduced expression in wheat silenced with TabZIP2, but it is not significantly different to the expression levels in BSMV:00 treated wheat. It appears that, with this level of silencing, there is not a significant difference in TaWRKY41 expression when TabZIP2 is silenced (p values = 0.82 and 0.59 for BSMV:TabZIP2A and TabZIP2B respectively).

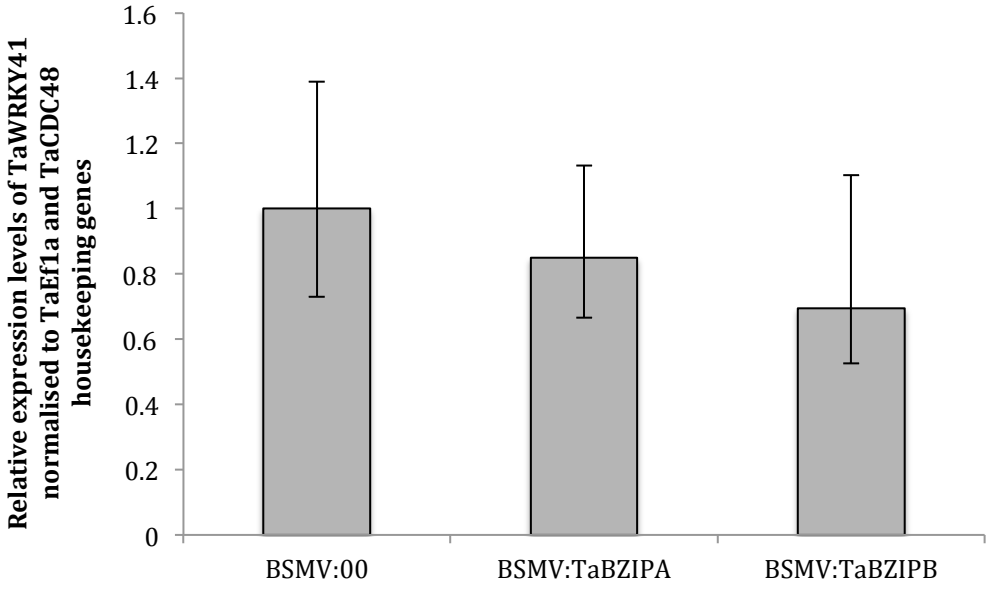

Figure 4.23: TaWRKY41 expression in TabZIP2 silenced wheat. qRT-PCR to measure TaWRKY41 expression in TabZIP2A and TabZIP2B silenced wheat compared to BSMV:00 control. RNA was extracted two weeks post silencing treatment (4-week-old seedlings). Leaves from three separate plants were collected per sample. qRT-PCR was performed using TaCDC48 and TaEF1a as housekeeping genes. This experiment was repeated three times. Two replicate wells for each sample were performed in the qRT-PCR. Error bars represent +/- 1 standard error.

TaWRKY63, however, shows a significant increase in expression in both the wheat samples treated with BSMV:TabZIP2A and BSMV:TabZIP2B (figure 4.24) (p values = 0.037 and 0.039 respectively). There is an increase, on average, of 2 and 2.6 times in BSMV:TabZIP2A and BSMV:TabZIP2B respectively.

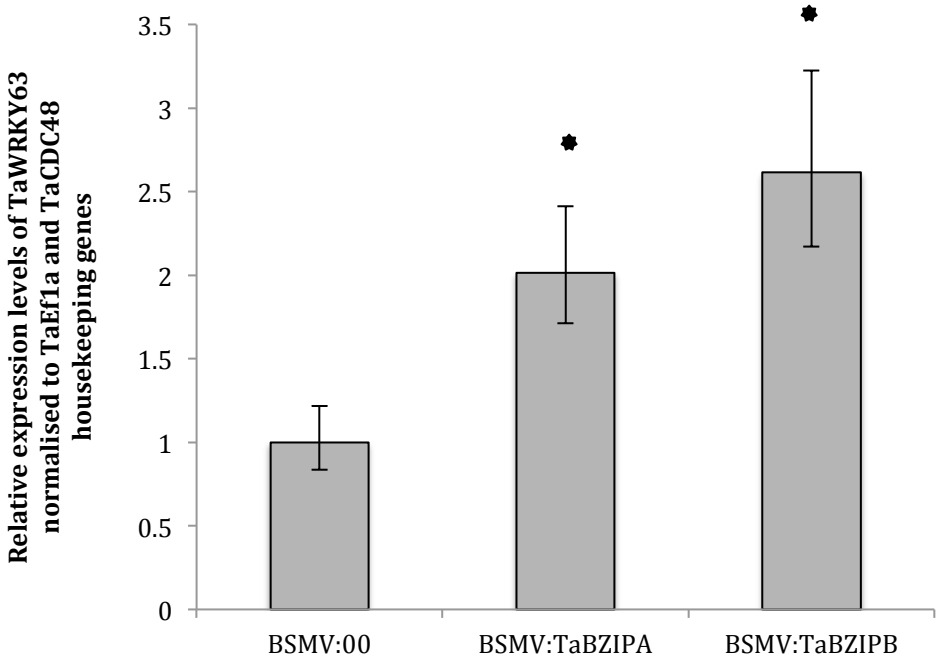

Figure 4.24: TaWRKY63 expression in TabZIP2 silenced wheat. qRT-PCR to measure TaWRKY63 expression in TabZIP2A and TabZIP2B silenced wheat compared to BSMV:00 control. RNA was extracted two weeks post silencing treatment (4-week-old seedlings). Leaves from three separate plants were collected per sample. qRT-PCR was performed using TaCDC48 and TaEF1a as housekeeping genes. This experiment was repeated 3 times. Two replicate wells for each sample were performed in the qRT-PCR. Error bars represent +/- 1 standard error. Asterisks are used to denote a results difference from the control of $p<0.05$.

TabZIP2 appears to act a negative regulator of both TaWRKY19 and TaWRKY63, although it does not regulate all TaWRKY genes.
WRKY genes are known to self regulate and regulate other WRKYs. With this knowledge we (Ari Sadanandom and myself) were interested to see whether TaWRKY19 regulated itself through TabZIP2. To do this the TaWRKY19

silenced wheat samples were used in qRT-PCR to measure the expression TabZIP2. This was to see if TaWRKY19 regulated TabZIP2, and, since previous data has shown that TabZIP2 regulates TaWRKY19, therefore itself (indirectly). Three repeats of the silenced wheat were used, repeating each sample twice in the qRT-PCR. The results of this can be seen in figure 4.25. There is a slight increase in expression of TabZIP2 in BSMV:TaWRKY19A. However it is not significantly different (p value = 0.69) and the error bars do cross over with the BSMV:00 control so there is no difference in the expression. TabZIP2 expression in BSMV:TaWRKY19B silenced wheat is no different to that in the BSMV:00 treated wheat control (p value = 0.79). This shows that although TabZIP2 regulates TaWRKY19, TaWRKY19 does not regulate TabZIP2.

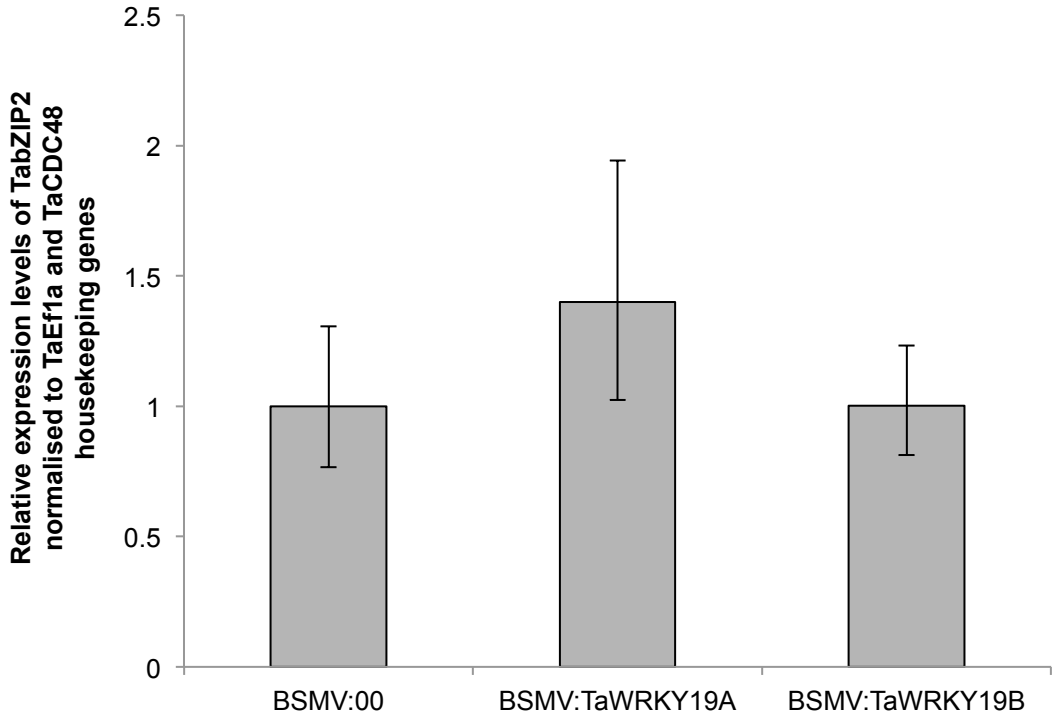

Figure 4.25: TabZIP2 expression in TaWRKY19 silenced wheat. qRT-PCR to measure TabZIP2 expression in TabZIP2A and TabZIP2B silenced wheat compared to BSMV:00 control. RNA was extracted two weeks post silencing treatment (4-week-old seedlings). Leaves from three separate plants were collected per sample. qRT-PCR was performed using TaCDC48 and TaEF1a as housekeeping genes. This experiment was repeated 3 times. Two replicate wells for each sample were performed in the qRT-PCR. Error bars represent +/- 1 standard error.

4.10 Investigating other TFs that potentially regulate TaWRKY19

The main focus was on TabZIP2 due to early results indicating that it was involved in wheat defence and the speed of cloning. This meant the other TaWRKY19 targets from the Y1H experiments were not investigated. Ideally it would have been informative to have silenced these other genes in wheat to test whether they were positive or negative regulators of TaWRKY19. I would also have liked to investigate if they had a role in defence against Septoria. However due to time constraints and cloning problems I was not able to perform these experiments.

Instead, as a preliminary investigation into the network of regulation of these TFs, qRT-PCR was used to elucidate whether TaWRKY19 regulates the targets therefore forming a potential feedback loop of regulation within the defence pathway. As before, primers were designed for each of the targets and tested to ensure they only amplified the gene of interest and tested their efficiency. Out of the five other targets, only two genes produced appropriate primers, TaTCP20 and TaHSFB1. Three repeats of TaWRKY19 silenced wheat samples were used with two well replicates in the qRT-PCR plate per sample. TaTCP20 expression does not show any significant difference in either of the TaWRKY19 silenced wheat lines when compared to BSMV:00 (figure 4.26). There is a slight reduction in the average expression in TaWRKY19A silenced wheat, however it is not significant (p values of 0.28 and 0.98 for BSMV:TaWRKY19A and BSMV:TaWRKY19B respectively).

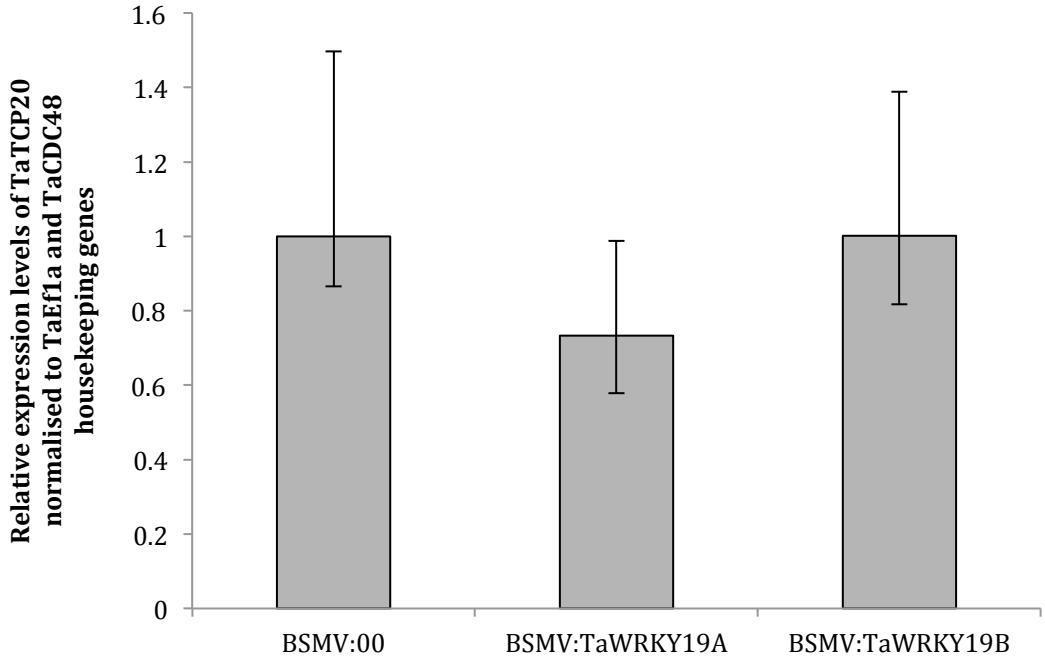

Figure 4.26: TaTCP20 expression in TaWRKY19 silenced wheat. qRT-PCR to measure TaTCP20 expression in TaWRKY19A and TaWRKY19B silenced wheat compared to BSMV:00 control. RNA was extracted two weeks post silencing treatment (4-week-old seedlings). Leaves from three separate plants were collected per sample. qRT-PCR was performed using TaCDC48 and TaEF1a as housekeeping genes. This experiment was repeated 3 times. Two replicate wells for each sample were performed in the qRT-PCR. Error bars represent +/- 1 standard error across the 2 biological replicates.

TaHSFB1, however, does show a difference in expression in TaWRKY19 silenced wheat lines (figure 4.27). In both TaWRKY19 silenced lines TaHSFB1 is, on average, more highly expressed than in the control line, however neither are significantly different compared to the control. In BSMV:TaWRKY19A silenced wheat, TaHSFB1 expression is upregulated (1.8 times on average, p value = 0.34). The expression of TaHSFB1 in BSMV:TaWRKY19B is not significantly upregulated (p value = 0.12), but has an average increase of 4.7 times over BSMV:00. This may reflect the levels of silencing across the two different samples (figure 3.9), with BSMV:TaWRKY19A have 38% reduction whereas BSMV:TaWRKY19B has a 45% reduction. Ideally a full knockout transgenic line would be used to

confirm all these results, but as previously mentioned these are difficult to generate.

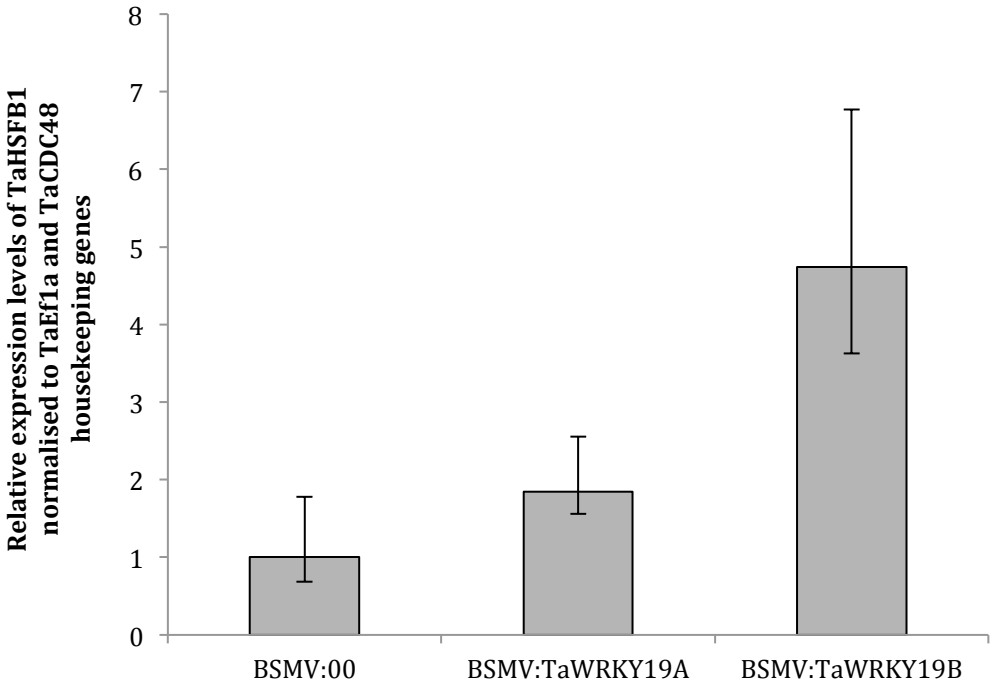

Figure 4.27: TaHSFB1 expression in TaWRKY19 silenced wheat. qRT-PCR to measure TaHSFB1 expression in TaWRKY19A and TaWRKY19B silenced wheat compared to BSMV:00 control. RNA was extracted two weeks post silencing treatment (4-week-old seedlings). Leaves from three separate plants were collected per sample. qRT-PCR was performed using TaCDC48 and TaEF1a as housekeeping genes. This experiment was repeated 3 times. Two replicate wells for each sample were performed in the qRT-PCR. Error bars represent +/- 1 standard error across the 2 biological replicates.

4.11 Conclusions

The aim of this chapter was to begin to elucidate the pathway upstream of TaWRKY19. To do this I used an Arabidopsis TF Y1H library[133] (approx. 1,200 TFs) to identify TFs that bound to TaWRKY19 promoter. The initial experiment showed 34 potential binding targets. After further experiments on higher levels of 3AT selection this was reduced to six TFs from five different families (HSF, TCP, bZIP, RING/U-box and Myb-like). Interestingly

two of these genes have a role in nitrogen (regulation and foraging for AtbZIP1[179] and AtTCP20 respectively). AtbZIP1 knockouts unusually show an inversion of gene expression in light and nitrogen conditions compared to WT. This indicates a genome wide regulation of genes involved in light and nitrogen by AtbZIP1[179]. Knockout mutants of AtTCP20 grow normal roots on none stressed media. However when placed on split media (half high nitrate, half low with the lateral root split over both media) the mutants showed no preference for high nitrate media, unlike WT plants that had preferential lateral root growth on high nitrate media. This indicates AtTCP20's involvement in nitrate foraging[198]. Nitrogen is regularly applied to crops to improve their growth and yield, however there is anecdotal evidence that the more nitrogen available, the more disease present upon the crop. Therefore, if a link between nitrogen, TabZIP2 and TaWRKY19 could be identified it may be useful for future breeding. This was one of the reasons TabZIP2 was focussed on for the rest of the work. TabZIP2 was also the first of the wheat homologues I managed to successfully clone allowing me to test TabZIP2s ability to bind to TaWRKY19 promoter. Both the Arabidopsis and wheat homologues bound to TaWRKY19 up to 40/60mM of 3AT selection pressure. They are not the strongest promoter interactors; however potential links to growth and PMT regulation meant TabZIP2 was focussed on.

After showing TabZIP2 could bind to TaWRKY19's promoter, we (Ari Sadanandom and myself) decided to investigate whether TabZIP2 had a stronger effect on wheat defence against Septoria. This was the original idea behind the Y1H experiment, looking to find a TF that had a larger effect on defence by going further upstream in the defence pathway towards pathogen perception. After successfully managing to silence TabZIP2, the silenced wheat was infected with Septoria. This experiment showed the opposite phenotype to TaWRKY19 silencing, with a delay in the onset of visible symptoms and a reduction in Septoria sporulation. There appeared to be no difference in the amount of pycnidia the Septoria produced. This could mean that whilst the Septoria was able to produce enough pycnidia they were not able to produce spores as efficiently when TabZIP2 is silenced, potentially using TabZIP2 in this process somehow. Further investigation with full

knockout plants should further elucidate the mechanism, by potentially allowing a subtler phenotype to be noticed.

TaWRKY19 and TabZIP2 have opposing roles in defence (positive and negative regulator respectively). TabZIP2 also binds to TaWRKY19's promoter region. Therefore the next step was to determine if TabZIP2 is a negative or positive regulator. To do this I used the TabZIP2 silenced wheat samples and measured expression of TaWRKY19. Based on this experiment I can postulate that TabZIP negatively regulates TaWRKY19 expression, either directly or indirectly. Further experiments including Chromatin Immunopreciptation sequencing (ChIP-seq) assay and DNA electrophoretic mobility shift (EMSA) assay would enhance the evidence from the Y1H experiment of TabZIP2s ability to bind TaWRKY19s promoter.

To investigate other potential targets of TabZIP2, I also measured the expression levels of other TaWRKYs – TaWRKY41 and TaWRKY63. I had originally planned on including measurements for TaWRKY2 and TaWRKY9 expression, however the results were inconclusive, with further experimentation needed. TaWRKY41 and TaWRKY63 are potential effector targets and (based on the guard hypothesis) have the ability to these perceive effectors and initiate a defence response, hence why I chose to study them. TaWRKY41 had no significant expression change in TabZIP2 silenced wheat. TaWRKY63, however, does change in expression when compared to the control, with higher expression when TabZIP2 is silenced. These genes offer more potential breeding target, consequently further experiments focussing on these genes would be highly interesting.

The final experiments in this chapter focus back on the other targets identified through the Y1H experiments. The aim was to see if there was a feedback loop in the TFs that bound to TaWRKY19, therefore beginning to identify a network of wheat defence. Unfortunately I only managed to design suitable primers for two of the five other targets, TaTCP20 and TaHSFB1. In TaWRKY19 silenced wheat TaTCP20 showed no difference in expression, so no feedback loop. However TaHSFB1 did appear to be regulated by TaWRKY19. With TaWRKY19 silenced wheat having higher levels of TaHSFB1 expression. Further experiments focussing on TaHSB1 and

TaWRWKY63 would be highly interesting and potentially offer more wheat defence breeding targets.

5. TaWRKY9 is a susceptibly factor against Septoria

5.1 Introduction

TaWRKY9 was chosen as target to investigate based on its homology towards AtWRKY11 (figure 5.1). Aligning the proteins using NCBI blast[150] shows they have high homology with an expected value of 5e-93 and an identity score of 49%. In knockout AtWRKY11 plants there is enhanced resistance against the bacterial pathogen Pseudomonas syringae pv tomato (Pst). AtWRKY11 is upregulated early in infection (0-4 hours post infection)[199].

AtWRKY11 expression response to chitin (PAMP from fungi) has been studied. This study is more relevant to my research being a fungal, rather than bacterial, pathogen response. Its expression was upregulated after treatment with chitin (30 minutes post infiltration). Interesting different splice variants of AtWRKY11 had differences in expression during early treatment, with the authors suggesting that the differential splicing is due to AtWRKY11 potentially being a key regulator of defence[200].

```
AtWRKY11      MAVDLMGCYAPRRADDQLAIQEAATAGLRSLEMLVSSLSSSSQAAGAHKASPQQQPFGEI      60
TaWRKY9       MAVDPMGCYTPRRADDQLAIQEAATAGLRSLELLVSSLSGAAPSK--APQQHLQQPFGEI      58
              **** ****:**********************:******.:: :      .  *******

AtWRKY11      ADQAVSKFRKVISILDRTGHARFRRGPVESSAPAAPVAAAPPPPPPPPAPVAA-------      113
TaWRKY9       ADQAVSKFRKVISILDRTGHARFRRGPVQSPTPPPPAPVAPPPPPPRPLAVVEPARPAPL      118
              ****************************:* :*  *. .******* *  *.

AtWRKY11      --------ALAPTSSQPQTLTLDFTKPNLTMSAATSVTSTSFFSSVTAGEGSVSKGRSLL      165
TaWRKY9       TAVAPVSVAAPVPLPQPQSLTLDFTKPNLTMSGATSVTSTSFFLSVTAGEGSVSKGRSLV      178
                       *      ***:*************.********** ***************:

AtWRKY11      SSGKPPLSGHKRKPCAGGHSEATANGGRCHCSKRRKNRVKRTIRVPAISSKIADIPPDEY      225
TaWRKY9       SAGKPPLSGHKRKPCAGAHSEANTTGSRCHCSKRRKNRVKTTVRVPAVSAKIADIPPDEY      238
              *:***************.****.:.*.************* *:****:*:**********

AtWRKY11      SWRKYGQKPIKGSPYPRGYYKCSTVRGCPARKHVERATDDPAMLVVTYEGEHRHTPGPLP      285
TaWRKY9       SWRKYGQKPIKVSPYPRGYYKCSTVRGCPARKHVERALDDPAMLVVTYEGEHRHSPGPMP      298
              *********** ************************ ****************:***:*

AtWRKY11      APPAAAAVA------AMPVSVAVSTGNGHV    309
TaWRKY9       MQMAPSPVPIPMPMGAPVAVASVSAGNGHV    328
                 * : *       *  . .:**:*****
```

Figure 5.1: Figure 5.12: Protein alignments of AtWRKY11 and TaWRKY9. The alignments were made using Clustal Omega[195,196].

I performed initial experiments to study TaWRKY9s expression in healthy and Septoria infected tissue using semi-quantitative PCR (figure 5.2). TaWRKY9 was upregulated in these experiments, with the highest expression (8dpi) occurring around Septoria's switch from biotrophic to necrotrophic growth.

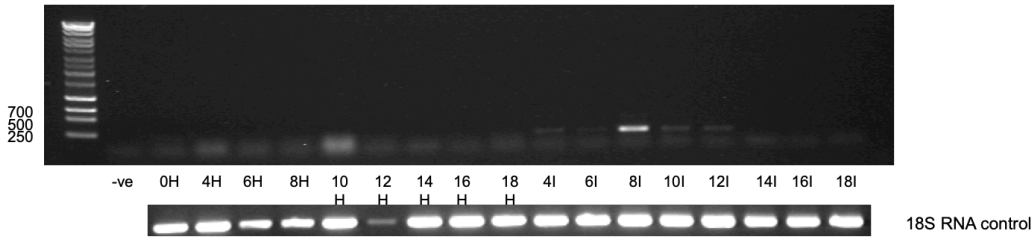

Figure 5.2: Gel of PCR showing TaWRKY9 expression in healthy and Septoria infected wheat over a time course of 0-18dpi. A housekeeping gene (18S RNA) was also run to assess concentration and quality of cDNA. A negative control containing no DNA is present in lane 2.
PCR products were run on a 1% agarose gel in 1xTAE buffer for size separation and visualised under UV light with quantity one software. A 1kb plus hyperladder (bioline).

From this we (Ari Sadanandom and myself) decided to further study TaWRKY9 in the hope that, like its arabidopsis homologues, it would be involved in defence, therefore making it a good breeding target.

5.2 TaWRKY9

The NCBI protein blast tool[150] was used to determine any protein domains present in TaWRKY9. The results of this blast can be seen in figure 5.3. TaWRKY9 is 328 amino acids long, with a weight of 34.64kDa. TaWRKY9 is a member of IId group of WRKYs (figure 3.1). The WRKY domain is located near the C terminal region of the protein. Babu et al[201] defined the other domain present, Plant_zn_clust, noting that this particular Zinc finger is associated with WRKYs TFs but also has high folding similarity to certain other TFs (Glial Cell Missing 1 and potentially transposases with the MudR-type transposase domain) across the animal and fungal kingdoms. They used WRKY TFs zinc finger region as a model to study the evolutionary history of

other TFs, hence why this domain has been defined separately in NCBI. I am not sure why it is located before the WRKY domain since the zinc finger of a WRKY domain is located towards the N terminal. This zinc finger is the most similar domain to a WRKY TFs in the animal kingdom[201].

A.

MAVDPMGCYTPRRADDQLAIQEAATAGLRSLELLVSSLSGAAPSKAPQQHLQQPF
GEIADQAVSKFRKVISILDRTGHARFRRGPVQSPTPPPPAPVAPPPPPPRPLAVVEP
ARPAPLTAVAPVSVAAPVPLPQPQSLTLDFTKPNLTMSGATSVTSTSFFLSVTAGE
GSVSKGRSLVSAGKPPLSGHKRKPCAGAHSEANTTGSRCHCSKRRKNRVKTTVRV
PAVSAKIADIPPDEYSWRKYGQKPIKVSPYPRGYYKCSTVRGCPARKHVERALDDP
AMLVVTYEGEHRHSPGPMPMQMAPSPVPIPMPMGAPVAVASVSAGNGHV

B.

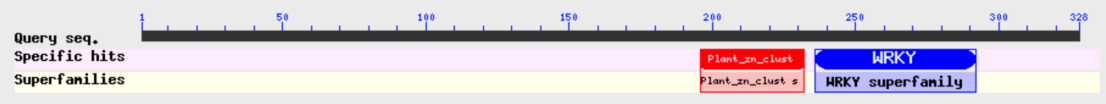

Figure 5.3: Protein sequence of TaWRKY9. A. Protein sequence of TaWRKY9 (328aa). Highlighted in red are the WRKY sequences of the WRKY domain. Highlighted in blue are the zinc finger sequences of the WRKY domain. B. Cartoon representation showing the position of the WRKY domains relative to the protein. This was generated using NCBI protein blast tool[150].

As with TaWRKY19, a full DNA sequence data was not available for TaWRKY9, only the CDS from the start codon until the stop codon was defined. The plan was to perform silencing experiments leading to Septoria infection experiments. Therefore, the first step was to sequence the 5' and 3' UTR to ensure the generation of silencing fragments that were specific to TaWRKY9[121].

Since both TaWRKY19 and TaWRKY9 were upregulated after Septoria infection, the same 5' and 3' RACE cDNA produced using RNA isolated from Septoria infected wheat leaves 8dpi was used. Three sets of nested primers were designed against the CDS region of TaWRKY9 so that they would amplify outwards from the gene, using the Universal primer mix (Clonetech) as the opposing primer. Using Q5 high fidelity DNA polymerase multiple

nested PCR reactions were performed (diluting the previous PCR 1/10 to be used as a template for the next PCR reaction). The PCR reactions were run on a 1% agarose gel (data not shown) and any resulting DNA bands were isolated. The DNA was extracted from the gel and sequenced. Unfortunately the PCR reactions for the 5' UTR failed, however the sequence for the 3' UTR was obtained. The results of the sequencing reaction can be seen in figure 5.4, which shows the DNA sequence of TaWRKY9 from the start codon until the poly A tail of the mRNA. The 3' UTR is highlighted in grey with the silencing fragment highlighted on top of this in blue. The 3' UTR is 132bps long. A potential sequence for the 5' UTR was eventually identified using JBrowse[202] and tracks from published RNA sequencing experiments. This is also highlighted in grey, with a size of 47bps.

Agcgagccaagatctgcagagtcacaggcgacctcacaccggcgacc**atg**gccgtggaccccatgggctgct
acacccctcgccgcgccgacgaccagctcgccatccaggaggccgccaccgccggcctccgcagcctggagc
tcctcgtctcctccctctccggcgcggcgccgtccaaggcgccgcagcagcacctgcagcagccgttcggcga
gatcgccgaccaggccgtctccaagttccgcaaggtgatctccatcctcgaccgcaccggccacgcccgcttcc
gccgcggccccgtccagtcgcctaccccgcctcctccggctccggtcgctcctccgccgcccccaccgcgccct
ctggccgtcgtcgagccggccaggcccgctcccttgaccgccgtggcgccggtgtcggtggccgcccccggtcc
ctctcccgcagccgcagagcctgacgctggacttcaccaagccgaacctgaccatgtcaggcgcgacgtccgt
gacgtccacgtccttctttctctcggtgaccgccggcgagggcagcgtgtccagagggccgcagcctggtctcc
gccggcaagccgccgctgtccgggcacaagagaaagccgtgcgccggcgcgcactcggaggccaacaccac
cggcagccgatgccactgctccaagagaaggaagaaccgcgtgaagacgacggtgagggtgcccgcggtga
gcgcgaagatcgccgacatcccgccggacgagtactcgtggaggaagtacggccagaagcccatcaaggtat
ccccttacccacggggctactacaagtgcagcacagtgcgagggtgcccggcgcggaagcacgtggagcgcg
ccctggacgacccggcgatgctggtggtgacgtacgagggcgagcaccgccactcgccggggccgatgccga
tgcagatggcgccgtcgccggtgccgattccgatgccgatgggcgcgcccgtagccgtagctagtgtgtccgcc
ggcaacgggcacgtc**tga**cttagttaaatttttctttctttccccatttggttgggggtgcttcgttcgttcgctcg
caatctgtctgatgtccgtgtaaagaagaagaaagatcgtagagagacggagagggaaacttaatgccagag
atttttactacctcccgaaaaaaaaaaaaaaaaaaaaaaaaaa

Figure 5.4: DNA sequence of TaWRKY9. TaWRKY9 CDS sequence is 988bp long. Sequence data was extended using RACE experiments and database searches with RNA sequencing experiments integrated. The 3' UTR identified is 132bps long. A section of the 5' UTR was identified (47bps). The ATG start codon and TGA stop codon are in bold, UTR sequences highlighted in grey and silencing fragment TaWRKY9A highlighted in blue.

5.3 TaWRKY9 silencing using VIGS

After sequencing the 5'UTR, a silencing fragment was designed for the VIGS and Septoria infection experiments to study TaWRKY9. Ideally two silencing fragments would be designed per construct, however since TaWRKY9's UTRs are so small only one, against the 3' UTR, was designed. It can be seen in figure 5.4, highlighted in blue. It is 80bp in length, a little shorter than the optimal length but still long enough to silence TaWRKY9.

Table 5.1 shows the results of the KWS silencing fragment prediction software. It works by assessing each 22bp fragment potentially produced by DICER cleavage for their silencing potential, assigning them a score from 1-10 with 10 being the highest. TaWRKY9 has two fragments with a score of 9, and a further 3 with high scores (over 7). Therefore even though it is a small fragment it had potential to silence TaWRKY9 well.

Start (bp)	End (bp)	Score	GC%
44	66	9	50
45	67	9	50
14	36	8	45
41	63	8	45
38	60	7	40
52	74	6	55
54	76	6	50
13	35	5	45
34	56	5	35
35	57	5	35
43	65	5	45
47	69	5	55
56	78	5	45
23	45	4	40
40	62	4	40
37	59	2	35

Table 5.1: TaWRKY9A silencing fragment efficiency analysis. Software analysis of the potential 22nt fragments incorporated into the RISC complex ability to silence. Scored 0-10, with 10 representing a high level of silencing efficiency and 1 a low level.

As well as testing the silencing efficiency of the TaWRKY9A fragment, I also used the Earlham Institute blast tool[151] to assess the ability of TaWRKY9A fragment to only silence TaWRKY9. TaWRKY9 is located on the long arm of chromosome 2. As can be seen in figure 5.5, all three TaWRKY9 homologues (across the A, B and D genomes) can be silenced using TaWRKY9A silencing fragment. Only two other genes were identified in the blast search, however there homology (<21bp length homology) is not high enough to be silenced via the wheat hosts natural defence system. One thing to note is that the silencing fragments and UTR sequence data were obtained from the wheat variety cv. Avalon, whereas this blast search is against cv. Chinese spring. This explains the single nucleotide polymorphism (SNP) being present in all three of the blast searches (C to T, position 50). All the experiments were performed on cv. Avalon.

TaWRKY9 chromosome 2AL

```
TTCGTTCGCTCGCAATCTGTCTGATGTCCGTGTAAAGAAGAAGAAAGATCGTAGAGAGACGGAGAGGGAAACTTAATGCC
|||||||||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||
TTCGTTCGCTCGCAATCTGTCTGATGTCCGTGTAAAGAAGAAGAAAGATTGTAGAGAGACGGAGAGGGAAACTTAATGCC
```

TaWRKY9 chromosome 2DL

```
TTCGTTCGCTCGCAATCTGTCTGATGTCCGTGTAAAGAAGAAGAAAGATCGTAGAGAGACGGAGAGGGAAACTTAATGCC
|||||| | |||||||||||||||||| |||||||||||||||||||||| ||||||||||||||||||||||||||||||
TTCGTTGGTTCGCAATCTGTCTGATGCCCGTGTAAAGAAGAAGAAAGATTGTAGAGAGACGGAGAGGGAAACTTAATGCC
```

TaWRKY9 chromosome 2BL

```
TTCGTTCGCTCGCAATCTGTCTGATGTCCGTGTAAAGAAGAAGAAAGATCGTAGAGAGACGGAGAGGGAAACTTAATGCC
|||||| | || |||||||||||||||||||||||||||||||||||||||| |||||||||||||||||||||||||||||
TTCGTTGGTTCTCAATCTGTCTGATGTCCGTGTAAAGAAGAAGAAAGATTGTAGAGAGACGGAGAGGGAAACTTAATGCC
```

Gene of unknown function chromosome 3DL

```
GATGTCCGTGTAAAGAAGAAGAAAGATCGTA
||||||  ||  ||||||||||||||||||
GATGTCTATGCCAAGAAGAAGAAAGATCGTA
```

Gene of unknown function unscaffolded

```
GATGTCCGTGTAAAGAAGAAGAAAGATC
|||||| |||  ||||||||||||||||
GATGTCTGTGCCAAGAAGAAGAAAGATC
```

Figure 5.5: Blast search of TaWRKY9A silencing fragment. The Earlham Institute's wheat genome[151] was used to blast search the DNA sequences of TaWRKY9A. Parameters were set to identify homology of sequences over 16bps long, against the cv. Chinese Spring wheat genome sequence. This is based on the size of siRNA produced by DICER cleavage[104,105].

Once I had designed the silencing fragment, the net step was to clone it into the BSMVγ vector. I used cDNA from Septoria infected wheat (8dpi) to clone the fragment since this had been used in the RACE experiments to identify the 3' UTR. Primers to amplify the silencing fragment were designed and used in a PCR reaction with Q5 high-fidelity DNA polymerase. The PCR program consisted of 30 cycles, 55^{0}C annealing temperature and 30 seconds for the extension step for the variable conditions. The PCR product was run on a 1% agarose gel with a 1kb and 50bp ladder (lanes 1 and 2) to separate the DNA bands based on size. A band around 80bp (figure 5.6) was cut from the gel and extracted using a gel extraction kit.

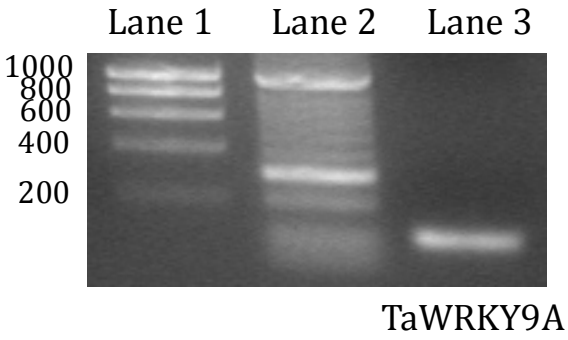

Figure 5.6: Gel of cloning PCR for TaWRKY9 silencing fragment. PCR products were run on a 1% agarose gel in 1xTAE buffer for size separation and visualised under UV light with quantity one software. A 1kb and 50bp hyperladder were used for size indication (lane 1 and 2 respectively). The expected size for TaWRKY9A (lane 3) was 80bp.

As in chapters 3 and 4, the fragment was cloned into the BSMVγ vector, sequenced and eventually transformed into *A. tumefaciens*. Two-week-old wheat plants were silenced with BSMV:TaWRKY9A. After two-weeks leaf samples from three plants were then collected and RNA extracted to check for silencing efficiency in TaWRKY9 plants. cDNA was synthesised from the RNA and diluted to 100ng ready for qRT-PCR. This experiment was repeated independently three times. Each well was replicated twice in the qRT-PCR. The results of the qRT-PCR to test the expression levels of TaWRKY9 in BSMV:00 and BSMV:TaWRKY9A are shown in figure 5.7. TaWRKY9 is silenced on average by 46% across the three experiments in wheat treated with BSMV:TaWRKY9A. There were higher levels of variation across the three experiments when compared to the previous silencing experiments. However the silencing is still statistically significant to a confidence of 95% ($p = 0.011$).

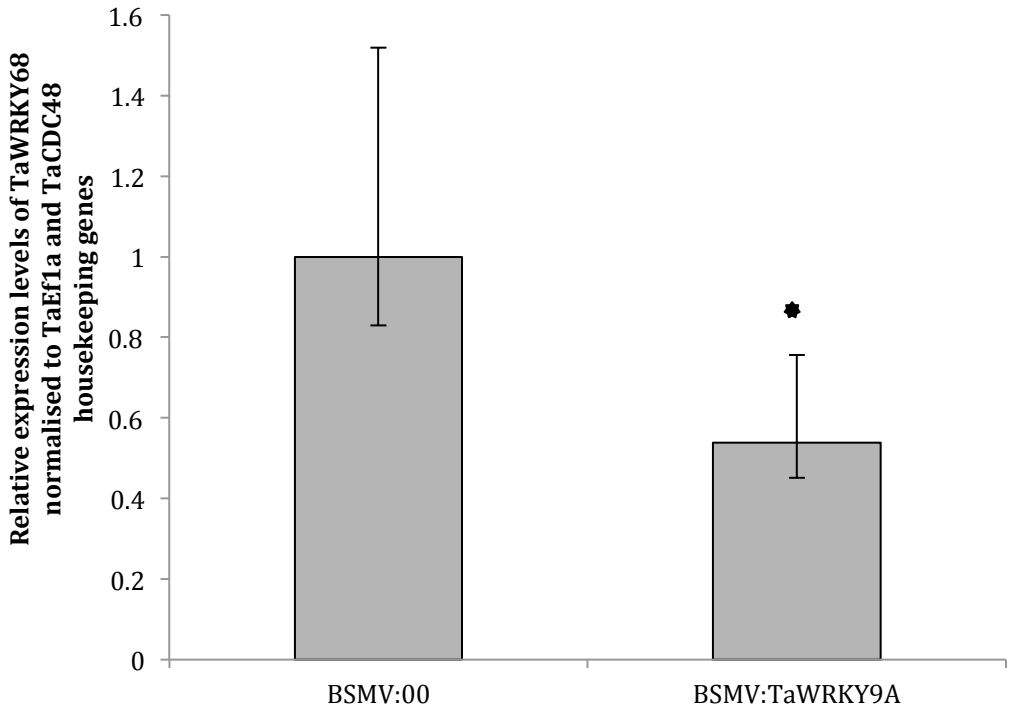

Figure 5.7: qRT-PCR to show silencing of TaWRKY9 in wheat. Fold change of TaWRKY9 in wheat silenced with BSMV:TaWRKY9A compared to BSMV:00 control. RNA was extracted 2 weeks post silencing treatment (4-week-old-seedlings). Leaves from three separate plants were collected per sample. qRT-PCR was performed using TaCDC48 and TaEF1a as housekeeping genes. This experiment was repeated 3 times. Two replicate wells for each sample were performed in the qRT-PCR. Error bars represent +/- 1 standard error. Asterisks are used to denote a results difference from the control of $p<0.05$.

5.4 TaWRKY9 in wheat defence against Septoria

After successful silencing of TaWRKY9 in wheat seedlings, the next step was to infect the plants with Septoria. Figure 5.8 shows a representative leaf from the three experimental repeats. Visible symptoms began at 12dpi in the control samples (BSMV:00), whereas in BSMV:TaWRKY9A treated plants the symptoms begin at 13dpi, a one day delay. Symptoms develop at the same rate within both sets of plants, taking 9 days for the plant tissue to die along

the whole length of the leaf. These experiments were performed along side experiments on TaWRKY19 silenced wheat. Thus I have used the same pictures for BSMV:00 symptom development as they were all performed at the same time.

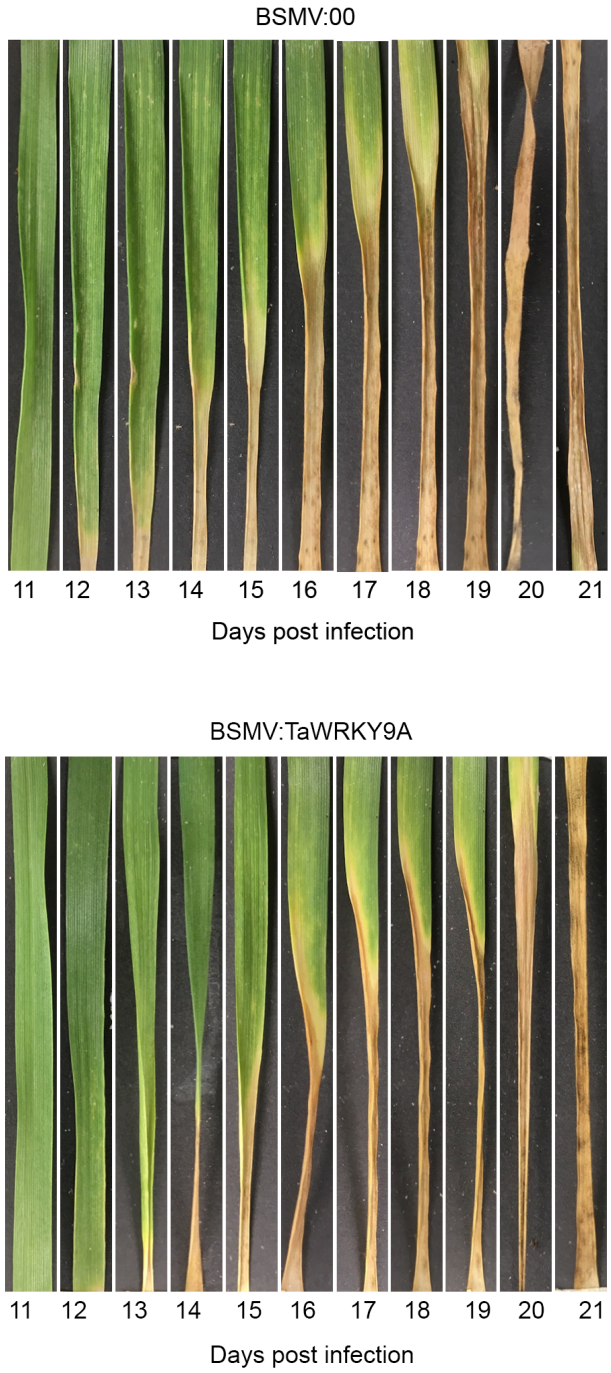

Figure 5.8: Septoria infection symptoms on TaWRKY9 silenced wheat. Four-week-old-seedlings, which undergone silencing treatment (BSMV:00, BSMV:TaWRKY9A) were stuck down onto black card and infected with Septoria (abaxial and adaxial). The seedlings were grown under high humidity conditions to encourage Septoria infection. The infection was then followed daily, with photos taken between 2-3pm. In this figure the initial stages of the infection symptoms are shown, from 11dpi until 21 dpi. Photos are representative of the symptoms seen in 3 independent experiments.

The pycnidia were then counted along a 2cm leaf length for each of the infected leaves. Figure 5.9 shows the results of the pycnidia counts. Wheat with TaWRKY9 silencing had, on average, a 35% reduction in pycnidia compared to the control samples. The difference is statistically significant to 95% confidence with a p value of 0.019.

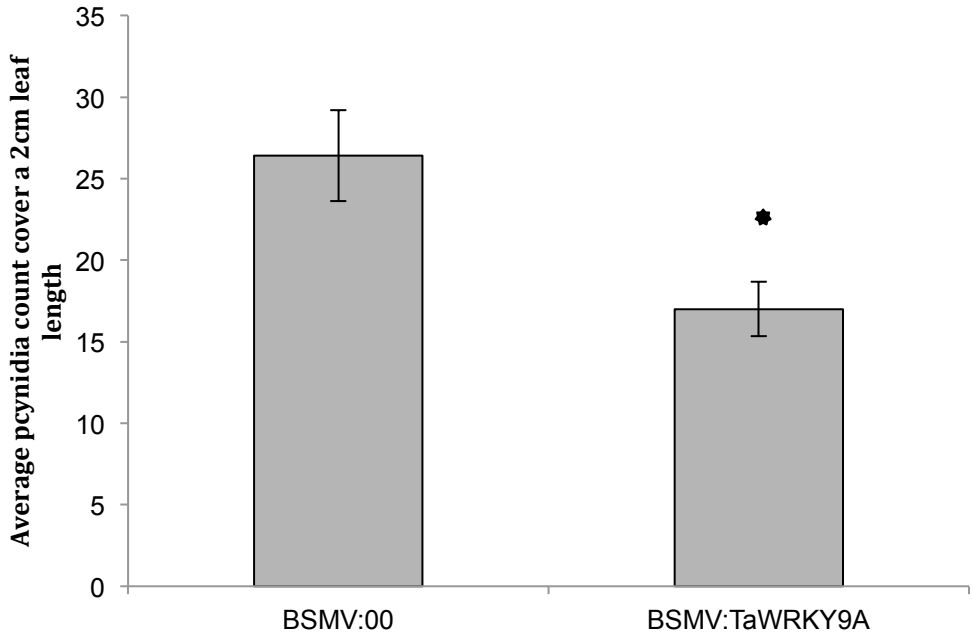

Figure 5.9: Septoria pycnidia count from infection upon TaWRKY9 silenced wheat. Pycnidia were counted over a 2cm leaf length. This experiment was repeated 3 times, with 5 leaves per experiment counted. Asterisks are used to denote a results difference from the control of $p<0.05$.

The spores were then counted. Unsurprisingly there is also a reduction (51%) in the number of spores Septoria produced on wheat with TaWRKY9 silenced compared to the BSMV:00 treated control (figure 5.10). This is statistically significant with over 99% confidence ($p = 1.33E-05$).

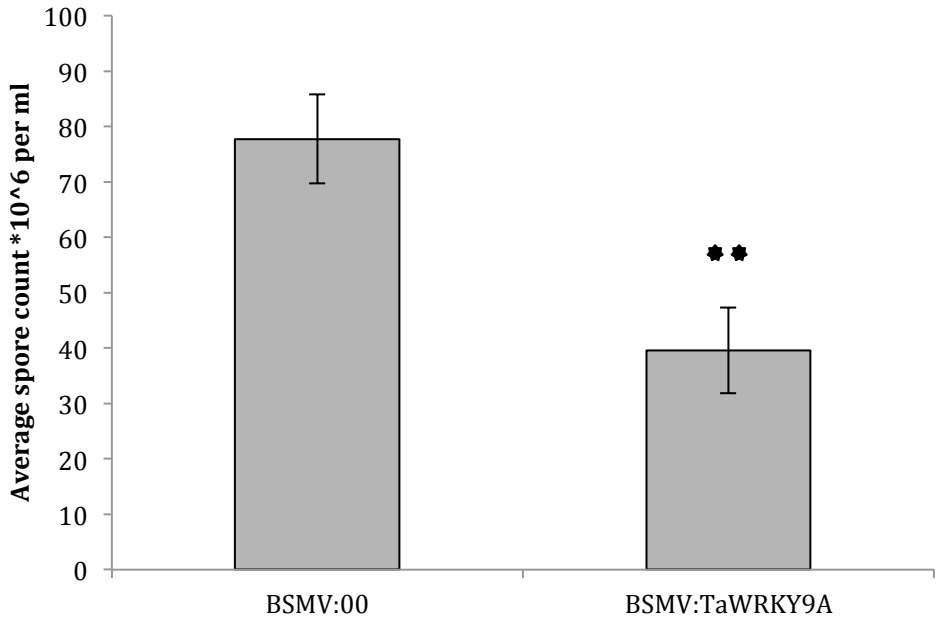

Figure 5.10: Septoria spore counts from TaWRKY9 silenced and infected leaves. Five leaves were submerged in 10ml of water and vortexed to wash the spores from the pycnidia. Spores were then counted using a haemocytometer under a light microscope (x10 magnification). Four spores counts across independent 4x4 squares on the haemocytometer were performed per spore suspension. Error bars represent +/- 1 standard error. This experiment was repeated independently 3 times. Double asterisks are used to denote a results difference from the control of $p<0.01$.

To determine the potential influence that modulating TaWRKY9 expression could have on plant defence, the relative pycnidia and spore reduction per 1% of silencing was calculated. Per 1% of silencing there is a 0.39 reduction in the pycnidia count. For the spore count it is more marked, with each 1% of silencing leading to a 0.65 ($*10^6$) reduction in the average spore count per ml. Together these results indicate that TaWRKY9 is a negative regulator of defence with silencing either leading to increased defence response or a decrease in Septoria's ability to infect.

5.5 TaWRKY9 promoter

To further investigate TaWRKY9 and build up a network of defence I planned to perform a Y1H against the same Y1H TF library as used in chapter 4. Y1H

assays allow for the identification of TFs that potentially regulate TaWRKY9, leading to possible breeding targets.

Firstly, TaWRKY9's promoter region was identified using databases provided by KWS and the Earlham Institute[151].. The aim was to identify the sequence 2kb upstream of the ATG start codon (supplemental figure 6). Using the databases and primer design tools[203,204], I designed primers to amplify a region of 1,973bp just upstream of the start codon (partially including the predicted 5' UTR sequence).

I used PlantPAN promoter analysis software[158] to predict potential TFs that would bind to TaWRKY9 promoter. This program utilises information from previously published interactions in different plant species (I used Arabidopsis, rice and maize) to predict the binding sites on the input sequence. Table 5.2 shows the results of this search. The most represented binding site is for WRKY TFs. It is known that WRKYs can regulate themselves and other WRKYs therefore this result is not surprising.

The At-Hook TF domain binds to the minor groove of AT rich DNA regions[205] and is present in many DNA binding proteins[206].

DNA-binding with One Finger (Dof) TFs are plant specific, they are mostly involved in growth and development (reviewed in [207]) and has been implicated in defence against pathogens[208].

The final TF with high numbers of predicted binding sites is the Squamose-promoter Binding Protein (SBP) family[209]. This is another plant specific TF family involved in developmental processes[210,211] and defence[212].

Family	No. of TFs
WRKY	28
AT-Hook	25
Dof	23
SBP	20
Myb/SANT	17
AP2;ERF	16
bZIP	11
GATA;tify	10
NAC;NAM	10
Others	8
Homeodomain ;HD-ZIP	8
AP2;B3;RAV	7
bHLH	7
TBP	7
Myb/SANT;MYB	6
SRS	6
TCP	6
B3	5
C2H2	5
Homeodomain ; bZIP ;HD-ZIP	5
Homeodomain ;HD-ZIP ;bZIP	5
Myb/SANT;MYB-related	5
B3;ARF	4
MYB-related	4
Myb/SANT;MYB;ARR-B	4
Alpha-amylase	3
GATA	3
Homeodomain ;TALE	3
MYB	3
MYB;ARR-B	3
AP2;B3	2
AP2;RAV;B3	2
bZIP;Homeodomain;HD-ZIP	2
Homeodomain	2
Homeodomain ;bZIP ;HD-ZIP ;WOX	2
HSF	2
Myb/SANT;ARR-B	2
AP2	1
AP2;RAV	1
BES1	1
CG-1;CAMTA	1
Dehydrin	1
E2F/DP	1
EIN3;EIL	1
ERF	1
FAR1	1
GATA;Dof	1
GRAS	1
HB-PHD	1
Homeodomain ;WOX	1
Homeodomain ;ZF-HD	1
LEA_5	1
LFY	1
MADF	1
Myb/SANT;G2-like	1
NF-YB;NF-YA;NF-YC	1
PsaH	1
Sox;YABBY	1
TCR;CPP	1
Trihelix	1
trp	1
VOZ	1
ZF-HD	1

Table 5.2: List of predicted TF that bind to TaWRKY9 promoter. DNA sequence for TaWRKY19 promoter was input into PlantPAN software [157,158]. TFs from the plant species Arabidopsis, rice and maize were selected for the analysis. Specific binding sites can be seen in supplemental table 5.

Using the designed primers, a PCR to clone the promoter was performed using HiFi DNA polymerase, 55^{0}C annealing temperature and 1-minute extension time for the PCR variables. The resulting PCR was run on a 0.8% agarose gel, which can be seen in figure 5.11. The ladder (lane 1) is not overly clear, however sequencing checks were performed confirming this to be the correct DNA band.

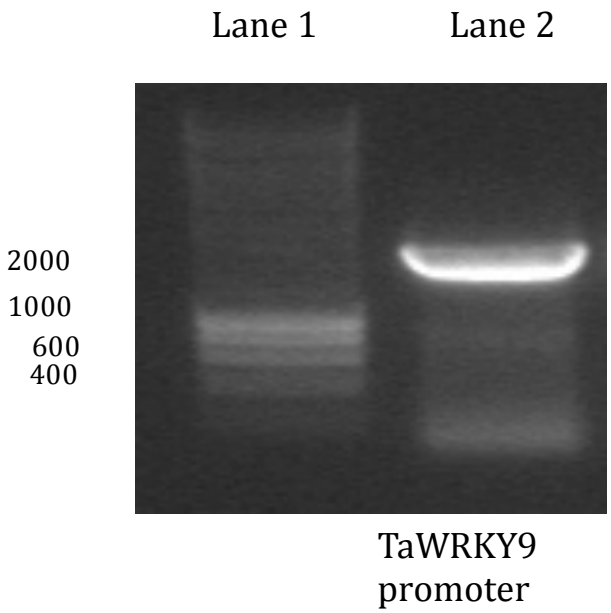

Figure 5.11: Gel of TaWRKY9 promoter cloning PCR. PCR products were run on a 0.8% agarose gel in 1xTAE buffer for size separation and visualised under UV light with quantity one software. A 1kb hyperladder was used for size indication (lane 1). The expected size for TaWRKY9 promoter (lane 2) was 1,973kb.

The band was excised from the gel and DNA extracted using a gel extraction kit before eventually transforming into PTUY1H (after confirming the sequence). Positive transformants were selected for by growing on minimal SD base media with amino acid mix –W.

5.6 Identifying TFs that bind to TaWRKY9 promoter

The same library was used for this experiment as in chapter 3. I did not succeed in reviving all the colonies from the glycerol stocks. The colonies that

grew can be seen in figure 4.1, with a 68% revival. Unfortunately the plate containing a large number of WRKY TFs (plate 10, 43 WRKYs) had a low level of revival (17%). As seen in table 5.2, WRKY TFs are predicted to be the most likely to bind to TaWRKY9 promoter so some strong interactors may have been missed due to the poor level of plate revival.

To measure the appropriate concentration of 3AT to include in the initial Y1H library screen the TaWRKY9 promoter yeast was mated with pDEST22 yeast. The mated culture was plated onto minimal SD base agar media with the following amino acid mix selection; –L-W, -L-W-H and –L-W-H +3AT (10, 20, 40, 60, 80 and 100mM). The plates were then incubated for 3 days in 28^{0}C. Following this experiment 20mM 3AT was chosen for the library screen as this concentration was the cut off for growth of the negative control mated yeast.

The Y1H library was mated with the TaWRKY9 promoter containing yeast, following the same procedure as in chapter 4.3, and allowed to grow in selection media (minimal SD base media with an amino acid mix –L-W). The mated cultures were then stamped (5µl each) onto minimal SD base agar plus amino acid mix plates with the following selection –L-W, -L-W-H and –L-W-H +20mM 3AT.

On these plates 62 wells grew across each of the selection pressures. The wells, gene ID and a brief description of the wells that grew are seen in figure 5.10. The family most represented in this screen are from the MADS family (table 5.3). There are number of WRKY TFs that have also bound. These were the most predicted TF from the software and may have been more represented had the colonies on plate 10 been successfully revived. There were almost double the amount of TFs bound to TaWRKY9 as opposed to TaWRKY19 promoter (34 TFs) (supplemental figure 7).

well	Gene ID	Gene description	No.	-L-W	+20mM	+40mM	+60mM	+80mM	+100mM
p1 a1	At2g45650	MADS (AGL6/RSB1)	1						
p1 a2	At4g11880	MADS (AGL14/XAL2)	2						
p1 a4	At3g57230	MADS (AGL16)	3						
p1 a5	At2g22630	MADS (AGL17)	4						
p2 a6	At3g57390	MADS (AGL18)	5						
p1 b2	At5g23260	MADS (AGL32/TT16)	6						
p1 c12	At1g60300	NAC	7						
p1 d1	At3g54990	AP2/ERF (SMZ)	8						
p1 d4	At4g36920	AP2/ERF (AP2/FI1/FLO2)	9						
p1 d5	At5g41315	bHLH (GL3/MYC6.2)	10						
p1 e5	At5g53420	CO-like	11						
p1 f9	At5g66160	RING-H2 (RMR1)	12						
p1 g7	At2g18670	C3HC4	13						
p1 h6	At2g31220	bHLH (BHLH010)	14						
p2 a7	At4g35040	bZIP (bZIP19)	15						
p2 c7	At1g12980	AP2/ERF (ESR1)	16						
p2 d5	At3g56980	bHLH (bHLH39/ORG3)	17						
p2 e1	At3g15170	NAC (NAC054)	18						
p4 a1	At1g75510	IIF factor beta subunit	19						
p4 a2	At5g43290	WRKY (WRKY49)	20						
p4 a3	At3g52270	IIF factor beta subunit	21						
p4 a4	At4g08250	GRAS (SCL26)	22						
p4 b1	At2g29060	GRAS (SCL33)	23						
p4 b3	At1g66350	GRAS (RGL1)	24						
p4 b4	At1g66350	GRAS (RGL1)	25						
p4 b5	At3g50650	GRAS (SCL7)	26						
p4 c3	At3g13840	GRAS (SCL29)	27						
p4 c5	At4g31800	WRKY (WRKY18)	28						
p4 d2	At5g43290	WRKY	29						
p4 d7	At1g29280	WRKY	30						
p4 g3	At4g17920	C3HC4 (ATL29)	31						
p4 g5	At5g03510	C2H2 type zinc finger	32						
p5 a1	At4g17490	AP2/ERF (ERF6)	33						
p5 a2	At2g38340	AP2/ERF (DREB2E/DREB19)	34						
p5 d4	At1g18710	MYB (MYB47)	35						
p5 d6	At3g13040	G2-like (PHL6)	36						
p5 e1	At3g09230	MYB (MYB1)	37						
p5 g6	At1g73410	MYB (MYB54)	38						
p6 a1	At2g31380;A	CO-like (BBX25/STH1;BBX31/MIP1B)	39						
p6 a6	At2g31380	CO-like (BBX25/STH1)	40						
p6 b2	At5g05770	Homeobox (WOX7)	41						
p6 c1	At5g53980	Homeobox (HB52)	42						
p6 c2	At1g26960	Homeobox (HB23)	43						
p6 c4	At2g36610	Homeobox (HB22)	44						
p6 c5	At2g18550	Homeobox (HB21)	45						
p6 c6	At2g18550	Homeobox (HB21)	46						
p6 d4	At1g02065	SBP (SPL8)	47						
p6 e4	At1g78600	CO-like (BBX22/STH3)	48						
p6 e7	At4g39070	CO-like (BBX20)	49						
p6 e9	At2g46830	MYB (CCA1)	50						
p6 f1	At4g24060	DOF (DOF4.6)	51						
p6 f4	At2g28920	C3HC4	52						
p6 h5	At1g29160	DOF (DOF1.5/COG1)	53						
p7 e1	At3g16500	AUX/IAA (IAA26/PAP1)	54						
p7 g2	At4g01550	NAC (NAC69/NTL13)	55						
p7 h1	At3g04420	NAC (NAC046)	56						
p7 h3	At3g56530	NAC (T5)19_180/NAC064)	57						
p8 a5	At1g35515	MYB (MYB8)	58						
p8 b8	At2g24840	MADS (AGL61)	59						
p8 b12	At5g63470	CCAAT-HAP5 (NFYC4)	60						
p8 c6	At2g03710	MADS (AGL3/SEP4)	61						
p8 d8	At5g38620	MADS (AGL73/MBB18.17)	62						
-ve control		TaWRKY68 promoter only	63						

Table 5.3: A representation of 62 TFs binding onto TaWRKY9 promoter through a Y1H experiment, with increasing selection. 62 TFs, identified through the initial Y1H experiment, were re-mated with TaWRKY9 promoter yeast and grown in liquid culture overnight. 5µl of this overnight was pipetted onto plates with minimal SD base media with an amino acid mix of either –L-W, -L-W-H, -L-W-H +3AT (20, 40, 60, 80 and 100mM). The plates grown for 3 days at 28^0C. Yeast that grew are highlighted in dark grey. TFs that grew on each selection are highlighted in light grey.

TF family	No. in Y1H
MADS	9
GRAS	6
Homeobox	6
NAC	5
Co-like	5
WRKY	4
MYB	4
AP2/ERF	3
bHLH	3
C3HC4	3
IIF factor beta subunit	2
DOF	2
RING-H2	1
bZIP	1
C2H2 type zinc finger	1
G2-like	1
SBP	1
AUX/IAA	1
CCAAT-HAP5	1

Table 5.4: TF family's represented in initial Y1H screening with TaWRKY9 promoter. A list of the families the 62 TFs that bind to TaWRKY9 promoter fall into.

To further reduce the targets, I repeated the experiment with the 62 targets but with increasing 3AT selection pressure (0-100mM). I began by mating the targets with TaWRKY9 promoter Y187α yeast, incubating them overnight at 28^0C before growing them in mating selection media (as in chapter 4). Pictures of the plates were then taken (supplemental figure 8) to assess the growth. The TFs with the highest binding strength (eight TFs) are shown in figure 5.11. They are also highlighted in light grey on table 5.3. I chose the eight strongest binders; these are (in no order) RMR1, bHLH10, IIF factor β subunit, SCL26, DREB19, MYB8, AGL3 and AGL73. All 8 of the TF's bind to TaWRKY9 promoter across each of the 3AT concentrations, indicating strong interactions. As can be seen in table 5.3, there are 18 other TFs that also bound strongly throughout the increasing 3AT concentrations. However to

reduce the number I chose the TFs that had the most growth across the three spots.

AmaGous-Like (AGL) 3 showed the strongest interaction, with multiple colonies in each of the spots across all the 3AT concentrations (figure 5.12). The next strongest interactor was the AGL73, which again has strong colony growth across all the concentrations of 3AT.

AGL3 and AGL73 are both members of the MADS box TF family. AGL3 is also known as SEPtallata4 (SEP4). AGL3/SEP4 is known to be involved in floral meristem regulation. Quadruple knockouts of SEP1/2/3/4, which are all closely related, have issues with their floral structure development[213]. Y1H do not indicate whether the TF binding would lead to activation or repression of the gene. However it is interesting to note that TaWRKY9 is regulated by TF involved in growth and development particularly those involved in floral development, which may potentially have an effect on wheat grain yield.

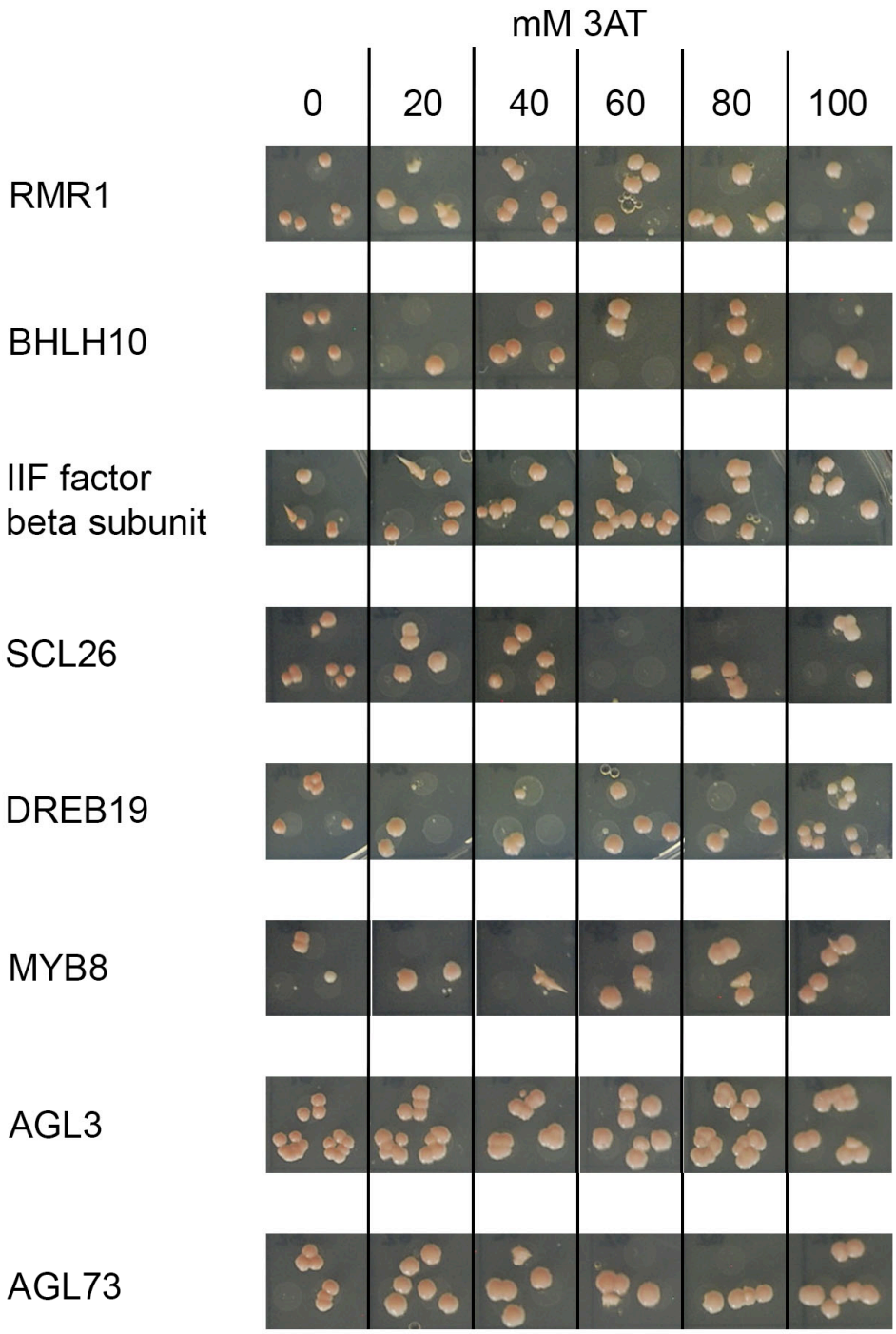

Figure 5.12: Y1H of strongest TFs binder to TaWRKY9 promoter. TFs that showed binding in the initial screen were re-mated with TaWRKY9 promoter and grown on increasing minimal SD base with amino mix –L-W-H and 3AT selection (20mM, 40mM, 60mM, 80mM and 100mM). Colonies were grown at 28^{0}C for 3 days. Pictures of the 62 targets were taken, included in the figure are the eight strongest TF binders to TaWRKY9 promoter.

As previously mentioned the Y1H library only contained Arabidopsis TFs. Therefore the next step was to identify the wheat homologues of the eight main targets. I used the KWS and Ensembl[214] databases to do this, using the blast functions on both databases to find the most up-to-date wheat genome sequence information.

Unfortunately I did not find homologues for each of the genes, this was partially due to time limitations using KWS database access. However, I was successful in finding homologues for DREB19, RMR1, SCL26 and bHLH010. When searching for a homologue for AGL3/SEP4 I ran into difficulties, I was able to find many homologues for SEP3 but none that were more closely related to SEP4 over SEP3. In Arabidopsis these genes do have strong homology, with similar expression patterns and functional redundancy however they do have some non-overlapping target genes[213,215]. An alignment of the closest wheat homologue for each of the four targets is seen in figure 5.13. The alignments were made using Clustal Omega software[195,196]. The identity score and expect value for each of the homologues can be seen in table 5.4. Identity scores are the percentage of amino acids in each position that are the same in both sequences. Expect values calculate the likely hood of an alignment occurring by chance elsewhere in a genome of a set size, the smaller the number, the more homology.

A.

```
AtDREB19      ------------------------------------------------------MEK-      3
TaDREB19      MTVDRKDAEAAAAAAAPFEIPALQPGRTCGAEESTRSHVLVKPIGSSNLPCNEYALLARQ      60
                                                                     : :

AtDREB19      EDNGSKQSSSASVVSS---RRRRRVVEPVEATLQRWEE---------EGLARARRVQAKG      51
TaDREB19      NPKGDAQ-PVASILRKKRPRRSRDGPNSVSETIRRWKEVNQQLEHDPQGAKRARKPPAKG      119
              : :*. *   **:: .   ** *   : *. *::**:*         :*  ***:  ***

AtDREB19      SKKGCMRGKGGPENPVCRFRGVRQRVWGKWVAEIREPVSHRGANSSRSKRLWLGTFATAA      111
TaDREB19      SKKGCMQGKGGPENTQCGFRGVRQRTWGKWVAEIREPN--------RVSRLWLGTFPTAE      171
              ******:*******  * *******.***********         * .******* **

AtDREB19      EAALAYDRAASVMYGPYARLNFPEDLGGGRKKDEEAESSGGYWL----------ETNKAG      161
TaDREB19      DAARAYDEAARAMYGALARTNFPVHPAQAPAVAVAAAIEGVVRGASASCESTTTSTNHSD      231
              :** ***.** .***  ** *** . . .      *  .*              .**::.

AtDREB19      NGVIETEGGKDYVVYNED-AIELG---------HDKTQNPMT-----------DNEIVNP      200
TaDREB19      VASSLPRQAQALEIYSQPDVLESTESVVLTPVEHYSHQDSVPDAGSSIARSTSEEDVFEP      291
               .    . .:   :*.:  .:*           * . *: :            ::::.:*

AtDREB19      A---VKSEEGYSYDRFKLDNGL--LYNEPQSSSYHQGGGFD-------------------      236
TaDREB19      LEPISSLPDG-ESDGFDIEELLRLMEADPIEVEPVNGGSWNGVEIGQQEPLYLDGLDQGM      350
                   .  :* . * *.::: *  :  :* . .  :**.::

AtDREB19      ------SYFEYFRF-------------------------    244
TaDREB19      LEGMLQSDYPYPMWISEDRAMHNPAFHDAEMSEFFEGL    388
                    * : *  :
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B.

```
AtSCL26       MNYPYEDFLDLFFSTHTDPLATAA-STSS-----NGYSLNDLDIDWD--------CDFRD       46
TaSCL26       MDVTMEDVVGDLEISGYSSISTSPSSSLDDGMGLYGWNALSPVADWGLFCSDDGGHDLHG       60
              *:   **.:. :  :  . ::*:  *: .      *:.  .   **.         *::.

AtSCL26       VIESIMGDEGAMMEPESEAVPMLHDQEGLCNSASTGLSVADG-------VSFGEPKTDES       99
TaSCL26       LIESMLCDDALIGKPDEHP-TMFT--DGPCYSNASDPSSTTTTNPGTPVQHDDTPDCNPE       117
              :***:: *:. : :*:..   *:   :* * * ::. * :            . *. : .

AtSCL26       KGLRLVHLLVAAADASTGANKSRELTRVILARLKDLVSPG----DRTNMERLAAHFTNGL       155
TaSCL26       KGLRLLHLLMAAAEALSGPHKSRELARVILVRLKEMVSSTSGNAGASNMERLAAHFTDAL       177
              *****:***:***:* :* :*****:****.***::**      . :**********:.*

AtSCL26       SKLLERDSVLCPQQHR-----DDVYDQADVISAFELLQNMSPYVNFGYLTATQAILEAVK       210
TaSCL26       QGLLDGSHSVAGTSRQAAMAASHHHSTGDVLTAFQMLQDMSPYMKFGHFTANQAILEAVA       237
              . **: .  :.  .::     .. :. .**::**::**:****::**::**.*******

AtSCL26       YERRIHIVDYDINEGVQWASLMQALVSRNTGPSAQHLRITALSRATNGKKSVAAVQETGR       270
TaSCL26       GDRRVHIVDYDLAEGIQWASLMQAMTSRPDGVSPPHLRITAITRSGGG--GARAVQEAGR       295
               :**:******: **:********:.**  * *  ******::*: .*  .. ****:**

AtSCL26       RLTAFADSIGQPFSYQHCKLDTN-AFSTSSLKLVRGEAVVINCMLHLPRFSH---QTPSS       326
TaSCL26       RLAAFAGSIGQPFSFGHCRLDSDERFRPATVRMVKGETLVANCILHQAAATTTVRRPTGS       355
              **:***.*******: **:**::  *  :::::*:**::* **:**    :    :  .*

AtSCL26       VISFLSEAKTLNPKLVTLVHEEV---------GLMGNQGFLYRFMDLLHQFSAIFDSLEA       377
TaSCL26       VASFLTGMASLGVKVVTVVEEEGEAEKNEEEASDAAAGGFVGRFMEELHRYSAVWDSLEA       415
              * ***:   :*. *:**:*.**          .  .  **: ***: **::**::*****

AtSCL26       GLSIANPARGFVERVFIGPWVANWLTRITANDAEVESFASWPQWLETNGFKPLEVSFTNR       437
TaSCL26       GFPTQSRVRGLVERVILAPNIAGAVSRAYR-GTDGEGRRGWGEWMRGSGFEMVPLSCFNH       474
              *:   . .**:****::.* :*. ::*    .:: *.  .* :*:. .**: : :*  *:

AtSCL26       CQAKLLLSLFNDGFRVEELGQNGLVLGWKSRRLVSASFWASCQTNQ--------------       483
TaSCL26       SQARLLLGLFNDGYTVEETRPNKIVLGWKARRLLSASVWAPLPLSVPSSPAEGVCQPMGM       534
              .**:***.*****: ***   * :*****:***:***.**    .

AtSCL26       ----------------------------        483
TaSCL26       APASGGFGRTEYDYVDSFLVEPAYALI        561
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C.

```
AtRMR1      -----------MRLVVSSCLLVAAPFLSSLLRVSLATVVLNSISASFADLPAKFDGSVTK        49
TaRMR1      MSPRRRPPLLLLAAVAVACACAAARP---------CAALVRLGAASFLDAPARFGPRVTG        51
                       :  *. :*  .**           .:.::.  :*** * **:*.  **

AtRMR1      NGICGALYVADPLDGCSPLLHAAASNWTQHRTTKFALIIRGECSFEDKLLNAQNSGFQAV        109
TaRMR1      DGICGSLRAAEPADACAPVRGAPGG----SGGMAFVLIARGNCSFEGKVRAAQRAGFDAA        107
            :****:* .*:* *.*:*:  * ..         *.** **:****.*:  **.:**:*.

AtRMR1      IVYDNIDNEDLIV-------------------------MKVNPQDITVDAVFVSNVAGE        143
TaRMR1      LVHDDEDKASLYSRDAHLVNGFGFRCLPSYDSKHTVGLGVVGDPEGIHIPAVFVSKMAGE        167
            :*:*: *: .*                           :  :*:.* : *****::***

AtRMR1      ILRKYARGRDGECCLNPPDRGSAWTVLAISFFSLLLIVTFLLIAFFAPRHWTQWRGRHTR        203
TaRMR1      TLKKFARGEDGECCINSSMDETAGTVLVMSFVSLVVIISVVASFLFARNCRLLRNGVDNR        227
             *:*:***.*****:*     :* ***.:**.**::*::.:   :** .     .* ..*

AtRMR1      TIRLDAKLVHTLPCFTFTD--SAHHKAGETCAICLEDYRFGESLRLLPCQHAFHLNCIDS        261
TaRMR1      PPYIKKHVVEKLPSVVYKAPCSSGNNCEEACAICLEDYDNGDMLRLLPCKHEFHVECIDP        287
               :. ::*..**...:.   *: ::. *:********  *: ******:* **::***

AtRMR1      WLTKWGTSCPVCKHDIRTETMSSEVHKRESPRTDTSTSRFAFAQSSQSR   310
TaRMR1      WLTKWGTFCPVCKLEVLTGG----------------------------   307
            ******* ***** :: *
```

D.

```
AtBHLH10      -MGCFDPNTPAEVTVESSFSQAEPPPPPPQVLVAGSTSNSNCSVEVEELS--EFH-----      52
TaBHLH10      MTGSL--------THDSSLA------P---------KCNDNTNIELQRFKVQSFSADILS      37
                *.:        * :**::      *         ..*.* .:*::.:.  .*

AtBHLH10      ----LSPQDCPQASSTPLQFHIN----PPPP-----PPPPCDQLHNNLIHQMASH-----      94
TaBHLH10      DSTNLSSEAARAINHLQHQLGIGLEQDMRPVETATWDTSICTIQDQIINHQLSEDPQNIL      97
                 ** : .   .    *: *.      *          *   .: : **::..

AtBHLH10      -QQQHSNWDNGYQDFVNLGPNSATTPDLLSLLHLPRCSLPPNHHPSSMLPT--------S      145
TaBHLH10      VQQQIQQYDA----ALYPNSGYTPAPDLLNLLH---CTVAPVFPPTASVFGDTALSGGTN      150
               *** .::*      :  . . : :****.***   *:: * . *:: :          .

AtBHLH10      FSDIMSS------SSAAAVMY--DPLFHLNFPMQPRDQNQLRN-------GSCLLGVEDQ      190
TaBHLH10      YLDLNDEFTGVAAIPDSGLMYTSDPALQLGYHAA--QSHALKDICHSLPQNYGLFPSEDE      208
              : *: ..         :.:**  ** ::*.:     :.: *::       .  *:  **:

AtBHLH10      I-QMDANGGMNVLYFEGANNNNGGFENEILEFNNGVTRKGRGSRKSRTSPTERERRVHFN      249
TaBHLH10      RDAILGVGSVGGDLFQDMD--DRQF-DTVLEGRRGKGDFGKGKG-KANFATERERREQLN      264
                 : . *.:.   *:. :  :  * : :** ..*    *:*.  . .  ****** ::*

AtBHLH10      DRFFDLKNLIPNPTKIDRASIVGEAIDYIKELLRTIEEFKMLVEKKRCGRFRSKKRARVG      309
TaBHLH10      VKYKTLRMLFPNPTKNDRASVVGDAIEYIDELNRTVKELKILVEQKWHGTNRRKIRKLDE      324
               ::  *: *:***** ****:**:**.** **::*:*:***:*  *  * * *

AtBHLH10      EGGGGEDQEEEEDTVNYKPQSEVDQSCFNKNNNNSLRCSWLKRKSKVTEVDVRIIDDEVT      369
TaBHLH10      EAAADG------ESSSMRPIRD----EQDNQLDGAIRSSWVQRRSRECHVDVRIVENEIN      374
              *....       :: . :*  :      ::: :.::*.**::*:*:  .*****:::*:.

AtBHLH10      IKLVQKKKINC-LLFTTKVLDQLQLDLHHVAGGQIGEHYSFLFNTKICEGSCVYASGIAD      428
TaBHLH10      IKLTEKKTTNSSLLHVAKVLDEFHLEIIHVVGGIIGDHYIFMFNTKVSEGSSIYACAVAK      434
              ***.:**. *. **..:****:::*:: **.** **:** *:****:.***.:**..:*.

AtBHLH10      TLMEVVEKQYMEAVPSNGY       447
TaBHLH10      RILQAVDAQHQALDIFN--       451
               :::.*: *:      *
```

Figure 5.13: Protein alignments of Arabidopsis Y1H targets with wheat homologues. Alignments are between the Arabidopsis targets and their closest wheat homologues, which were identified through database searches. The alignments were made using Clustal Omega[195,196]. A. AtDREB19 B. AtSCL26 C. AtRMR1 and D. AtbHLH010. A genome copies were chosen for the wheat homologues.

Arabidopsis homologue	Wheat homologue	Identity score	Expect value
AtDREB19	TaDREB19	60%	1e-37
AtRMR1	TaRMR1	39%	2e-68
AtSCL26	TaSCL26	43%	2e-126
AtbHLH010	TaBHLH010	34%	3e-51

Table 5.5: Identity score and expect values for Y1H targets wheat homologues. Identity scores and expect values of the Arabidopsis and wheat homologues were determined aligning the protein sequences using NCBI blast tool[150].

Ideally the next step would have been to clone the wheat homologues to see if they also bound to TaWRKY9s promoter and to what strength. I also planned to eventually design and clone silencing fragments for each of these genes, leading to silencing and Septoria infection experiments. However, I ran out of time to perform either of these experiments, instead focussing on the TaWRKY19 Y1H target TabZIP2 (chapter 4).

I did however begin investigating the network surrounding TaWRKY9. qRT-PCR primers were designed for each of the four wheat homologues. The plan was to study whether TaWRKY9 regulated these genes, showing a feedback loop in the system. These results are preliminary, having only been repeated twice. Two of the genes did not show any expression in either the BSMV:00 control plants or the BSMV:TaWRKY9A silenced plants (bHLH10 and SCL26). Figure 5.14 shows the results from the qRT-PCR for the wheat homologue of AtDREB19. From the two repeats it appears that TaDREB19 is highly upregulated in TaWRKY9 silenced wheat. This indicates that there may be a feedback loop within this interaction.

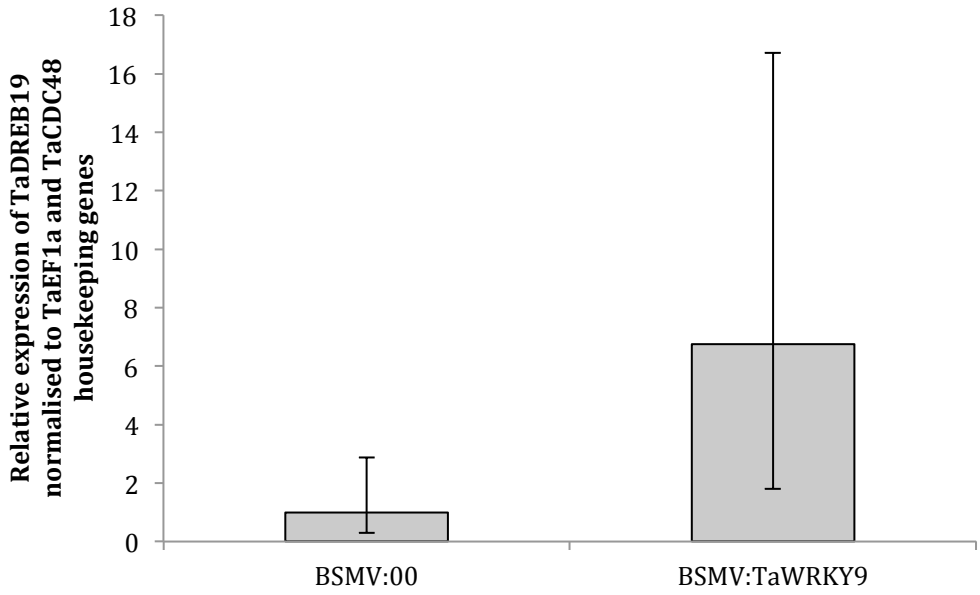

Figure 5.14: TaDREB19 expression in TaWRKY9 silenced wheat. Fold change of TaHSFB1 in wheat silenced with BSMV:TaWRKY9A compared to BSMV:00 control. RNA was extracted two weeks post silencing treatment (4-week-old seedlings). Leaves from three separate plants were collected per sample. qRT-PCR was performed using TaCDC48 and TaEF1a as housekeeping genes. This experiment was repeated 2 times. Two replicate wells for each sample were performed in the qRT-PCR. Error bars represent +/- 1 standard error.

Figure 5.15 shows the qRT-PCR results looking into the expression of TaRMR1 in TaWRKY9 silenced plants. Although there is a slight reduction in expression I do not think it will be significant after the third repeat is performed.

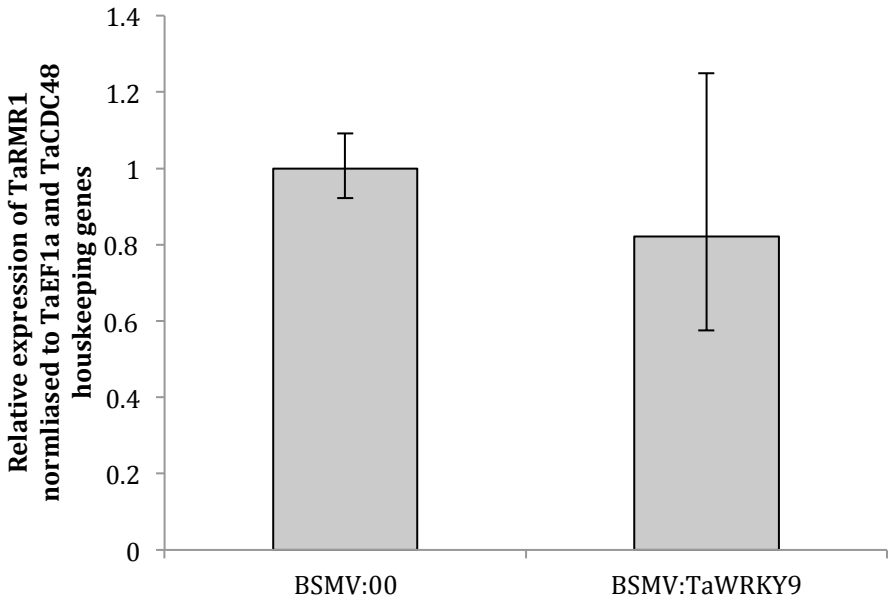

Figure 5.15: TaRMR1 expression in TaWRKY9 silenced wheat. Fold change of TaRMR1 in wheat silenced with BSMV:TaWRKY9A compared to BSMV:00 control. RNA was extracted two weeks post silencing treatment (4-week-old seedlings). Leaves from three separate plants were collected per sample. qRT-PCR was performed using TaCDC48 and TaEF1a as housekeeping genes. This experiment was repeated 2 times. Two replicate wells for each sample were performed in the qRT-PCR. Error bars represent +/- 1 standard error.

5.7 Conclusions

TaWRKY9 was first identified through investigating select WRKYs expression change after Septoria infection, with TaWRKY9 becoming upregulated around the onset of visible symptoms (12dpi). Previously studied Arabidopsis homologues of TaWRKY9 show a role in defence against bacterial pathogens through overexpressor and knockout studies[199,216]. This was the basis for studying TaWRKY9 in wheat defence against Septoria.
The first step was to design silencing fragments against TaWRKY9, however after performing RACE experiments to determine the UTR sequences, I could only design one fragment against the 3' UTR (due to sequence length). Ideally two silencing fragments are needed per gene, however blast studies of the silencing fragment showed it to be very specific towards TaWRKY9.
Through VIGS experiments I managed to silence TaWRKY9 in wheat seedlings, these plants were then used in Septoria infection assays. Infection

on TaWRKY9 silenced wheat led to a delay in the onset of visible symptoms, and a reduction in both Septoria pycnidia and spore production. These results imply that TaWRKY9 is a negative regulator of defence, with silencing increasing resistance against Septoria. Work presented in this chapter based on TaWRKY9 silencing and Septoria infection led to TaWRKY9 being patented for use in resistance variety wheat breeding in collaboration with KWS[162].

To help understand the mode of action for TaWRKY9 and build up a defence network, I performed a Y1H experiment with TaWRKY9 promoter against an Arabidopsis TF library. From this initial experiment 62 TF binders were identified from multiple different TF families. Further selection pressure resulted in this number being reduced to eight TFs, although a further 18 TFs also showed strong interactions. This is a greater number of TF binders when compared to TaWRKY19's promoter (chapter 4). Both experiments were carried out concurrently so there were no differences in the technical aspects of the experiment. A number of these TFs are involved in growth and development rather than defence. This is interesting, as the balance between growth and defence and decoupling this has been a strong research focus in my research group and others.

Ideally I would have followed up on each of the four wheat homologues identified for AtDREB19, AtRMR1, AtSCL26 and AtbHLH10, however my primary focus was on TaWRKY19 promoter binding TF, TabZIP2 (homologue of AtbZIP1, chapter 4). Preliminary investigations into the potential for a feedback loop between the four genes and TaWRKY9 were performed. I found that TaWRKY9 did appear to regulate the expression of TaDREB19 negatively; with TaWRKY9 knockdown plants having higher TaDREB19 expression compared to the control. This was not the case for TaRMR1, whose expression was unchanged in TaWRKY9 silenced plants. Unfortunately, until I can modulate the expression of each of these genes I do not know whether they positively or negatively regulate TaWRKY9.

Through this chapter I have identified multiple potential breeding targets, with particular focus on TaWRKY9. Further experiments are needed to study the TFs identified through the Y1H assay. However the potential to discover

multiple defence breeding targets that can be stacked to reduce Septoria resistance evolution is highly exciting.

6. Discussion

6.1 Introduction

Many WRKY TFs show differential expression after Septoria infection[138,217,218]. Other TFs families that also have expression changes post Septoria infection include bZIP and bHLH TFs[138]. Some well used Quantitative Trait Loci (QTLs) have been studied and linked to TFs[219], including a WRKY[219] and bHLH genes[220] involved in defence against *Fusarium graminearum* (causal agent of fusarium head blight). This knowledge led as the basis for the project, with an aim to find defence related breeding targets. The focus of the project was on WRKY TFs, with an aim to begin unlocking the wheat defence pathway against Septoria.

WRKY TFs are important in plants, not only defence but also other plant processes (reviewed in [68,221,222]. The wheat WRKY family is expanded in comparison to Arabidopsis (199 and 72[74] respectively). There is an uneven distribution of group expansion in the wheat WRKYs compared to Arabidopsis WRKYs, with an increase in group III WRKYs but a decrease in group I WRKYs. Currently there does not appear to be a clear trend between the structure of WRKYs and their function[152]. It is interesting that the groups have not expanded in the same proportions, in the future, after further investigation, a correlation may become clearer.

Preliminary experiments looking at the expression changes of 15 TaWRKYs in Septoria infected samples, lead to the identification of two genes whose expression were upregulated post infection, TaWRKY19 (chapter 3 and 4) and TaWRKY9 (chapter 5).

Whilst expression changes do show an indication of involvement in defence, protein levels and the expression levels of downstream gene targets of these WRKYs would offer more of an insight of how the change in WRKY expression levels effects the cell, and the defence response. Changes in expression are not the only level of regulation a cell can perform, with PTMs offering a rapid and dynamic method of control (reviewed in [223-226]). However the study of these concepts would involve the generation of transgenic wheat, which, in wheat, takes a considerable amount of time. Also

at the start of the study the targets of each of the WRKYs were unknown, hence why expression changes of the WRKYs were used in this study.

6.2 TaWRKY19

TaWRKY19 expression begins to become upregulated from 6dpi in the Septoria infected samples. The visible onset of symptoms occurs around 12dpi in my experiments, this occurs when the Septoria fungus begins to kill the leaf tissue as it switches from biotrophic to necrotrophic growth.

RNA sequencing experiments have shown a distinct difference in wheat gene expression patterns across the biotrophic and necrotrophic Septoria infection phase. It appears that Septoria actively attempts to down regulate defence related genes in early infection[218,227]. It would be interesting to see if TaWRKY19s expression pattern during infection differs across different wheat varieties and Septoria isolates. TaWRKY19's expression may become upregulated earlier or stronger in resistance varieties, which would enhance evidence of its involvement in the defence response.

Little is known about the trigger for Septoria's switch from biotrophic to necrotrophic growth. There are two current potential hypotheses, first that the switch occurs once the Septoria has reached a critical mass within the apoplastic space. This ensures the fungus is widespread enough to fight any defence response and rapidly kill the plant cells[4]. The other hypothesis implicates a balance of evading detection and necrotrophic infection; Septoria continues to grow until it senses that the host has detected it. Then before the host has a chance to mount an immune response, the fungus enters its necrotrophic phase and kills the cells[228]. The results in this thesis would suggest the former hypothesis to be true, with symptoms for wheat silenced with TaWRKY19 showing a day earlier than the control and increased susceptibility to Septoria. Indicating that somehow Septoria can grow faster without TaWRKY19.

However data from the infection time course would suggest that Septoria is detected before the onset of visible symptoms (figure 3.2). TaWRKY19's expression starts to become upregulated around 6dpi when compared to the healthy samples, with the expression continuing to increase up until 12dpi,

corresponding with the growth phase switch. So TaWKRY19 appears to be involved in early defence against Septoria, potentially working during early detection to fend off Septoria, hence why silencing causes an increase in susceptibility. Septoria can be measured by quantifying the total amount of Septoria DNA in the leaf. Whilst I did initial experiments to find suitable qRT-PCR primers, I did not follow up on this method. It would be useful to see if there is a correlation between the amount of Septoria infection and the switch to necrotrophic growth in different silenced and WT lines.

Wheat WRKY genes can have high homology towards each other, therefore it was decided that the silencing fragments should be designed against the UTRs of each gene[121]. Through RACE experiments I managed to sequence TaWRKY19s 3' UTR (192bps, figure 3.4), allowing the design of two silencing fragments against TaWRKY19. Ideally these silencing fragments would have been longer[110,111], however reports of fragments as small as 78bp have still shown high levels of silencing[229]. I also checked for the efficiency of the silencing fragments, with both showing at least one fragment with high levels of silencing potential (tables 3.2 and 3.3).

As well as predicting an acceptable level of silencing I also tested the silencing fragments in a blast search for any potential off target silencing. From this 2 off target genes were found with homology towards TaWRKY19A and one non-coding off target region found with homology towards TaWRKY19B. Further qRT-PCRs testing the expression levels of these off target genes/sequences is needed to fully ensure the infection phenotype is not due to the down regulation of these genes. However it should be noted that the genes that may have been silenced differed between the two fragments so, since plants silenced with both silencing fragments showed similar infection phenotypes, it indicates that the phenotype is due to TaWRKY19. Also neither fragment silenced each of the three homologues of any of the off target genes (although not all genes have copies across each genome).

VIGS was performed on 2-week-old seedlings using the two silencing fragments from TaWRKY19. The levels of silencing for the two fragments were 38% and 42% for BSMV:TaWRKY19A and BSMV:TaWRKY19B

respectively. TaWRKY19 expression was measured after two weeks of silencing treatment, however without Septoria infection TaWRKY19 has fairly low levels of expression (figure 3.2). On reflection it would have been better to measure TaWRKY19 expression in the Septoria infected leaves between 10-14dpi, giving a more accurate level of silencing. This would coincide with when TaWRKY19s expression was highest (figure 3.2) and give a more insightful view of the effect of TaWRKY19 has on Septoria's ability to infect wheat with TaWRKY19 modulated. Adding to this, papers have been published with similar levels of silencing so I was not discouraged by these knockdowns[45,119,137].

Resistance is categorised into qualitative and quantitative resistance. Qualitative resistance loci have a stronger phenotype (such as the QTL, *Stb6*[26,48,50]), however Septoria is more likely to evolve resistance towards the QTLs. They are also more likely to be variety and isolate specific[166]. Septoria is less likely to evolve resistance to quantitative resistance (which can be controlled by many genes in the same chromosomal location), but they only lead to small or moderate gains in resistance. Of the known qualitative QTL's there are none that cover the 2BS chromosome[230], the chromosomal location of TaWRKY19. There is a quantitative QTL located on 2BS (QTL3), which causes a 20% increease in resistance to Septoria and has yet to be assigned to a gene or set of genes[166]. Neither KWS nor myself investigated whether TaWRKY19 was the gene associated with QTL3.

During the PhD TILLING lines for TaWKRY19 and TaWRKY9 were acquired from NIAB. Lines were first selected based on the mutations ability to form a premature stop codon, leading to a knock out in the gene expression, however this was not always possible. KWS were working on crossing these lines so that there was a mutation across the A, B and D genome homologues. The hope was to use these plants in Septoria seedling infection assays, exactly the same as the VIGS lines, therefore confirming the VIGS and potentially seeing a stronger phenotype due to the reduced or knocked out expression of TaWRKY19 and TaWRKY9. However, due to the lengthy process of crossing and backcrossing, these lines were not available. I assume

that KWS will still use the lines in field trials to see if the results I have found in my laboratory experiments carry across to the field.

To further investigate TaWRKY19, I cloned the protein into pEARLEYGATE104, which produces recombinant protein with a YFP tag. The results showed that TaWRKY19 localises to the nucleus whereas YFP localises throughout the cell. Staining of the nucleus and other key cell components would enhance the validation of this experiment. TaWRKY19 nuclear localisation is unsurprising with it being a TF. Further work is needed to fully ensure that TaWRKY19 was expressed in the *N. benthamiana* plants and that the signal seen in these plants was due to TaWRKY19. Partial deletions and amino acid substitutions of the TaWRKY19 protein could show a difference in localisation. This is shown with AtWRKY6 whose novel nuclear localisation signal was identified after deletions and then substitutions of a region of 37 amino acids caused AtWRKY6 to localise throughout the cell as opposed WT, which localised to just the nucleus[231]. The nuclear localisation signal (NLS) found in AtWRKY6 is not present in TaWRKY19 (based on a blast search[150] of the NLS protein sequence against TaWRKY19 protein sequence).

On reflection I would have also liked to study TaWRKY19's expression with expression driven by it's own promoter as opposed to 35S promoter. However, due to TaWRKY19's low expression level, this may have led to little or no visualisation. I also only repeated the experiment once so cannot infer too much from this result, however TaWRKY19 expression has been seen in another paper in the nucleus[152]. Other studies on WRKY localisation have previously used the 35S promoter to drive expression[232,233].

Localisation changes under stress conditions have been previously seen in other TFs[234,235]. Therefore I would be interested to see if there are any TaWRKY19 localisation changes after fungal infection, potentially using *Botrytis cinerea* fungus, which has been used in our laboratory previously to infect *N. benthamiana* leaves (which were used in the localisation study). TaWRKY19 has been previously studied[152] using Arabidopsis plants that overexpressed TaWRKY19. The study concerned abiotic stresses, finding TaWRKY19 to be upregulated in cold, drought, salt and ABA treatments.

Plants overexpressing TaWRKY19 also conferred a tolerance to these stresses. In cold and salt stress there was an increase in soluble sugar content. Sugar is important in both abiotic and biotic stresses (reviewed in [236,237]). High sugar levels (as seen in Arabidopsis overexpressing TaWRKY19[152]) have been associated with an increase in defence related compounds[238]. Sugar is also an important signalling molecule (reviewed in [236,237]) and in priming defence (reviewed in [236]).

I did initial experiments to measure the levels of soluble sugar in the TaWRK19 silenced wheat leaves but I was unsuccessful. Ideally I would have followed up on this to see if the results from Can-Fang et al[90], who studied TaWRKY19 in Arabidopsis plants, could be replicated in wheat plants. Experiments investigating the expression of sugar reporter genes such as β amylase, whose expression is reduced in high sugar levels[239], in TaWRKY19 knockdown plants would also offer an insight into any potential link.

I identified the region 1,497bp upstream of TaWRKY19's ATG start codon and cloned this for use in a Y1H experiment. Selecting a set number of bps upstream is a suggested method for promoter analysis when the promoter has not been identified previously[240]. I aimed for 2,000bp upstream, however 1,500bp was satisfactory. At that point in time the wheat genome had not been fully assembled hence why I could only obtain the sequence 1,500bp upstream of the ATG for TaWRKY19.

I used the Arabidopsis RR TF library[133]. The TF library contained approximately 1,200 different TFs. I tried to revive the entire library, however some of the plates did not grow very well (figure 4.2). This included a large number of WRKY TFs on plate 10. Other members of my lab also work on WRKY TFs, with the Y1H experiments hvaing potentially identified the Arabidopsis homologues of these WRKYs, giving a clear link between two WRKYs studied within the group. Hwoever this was less likely due to the poor revival of yeast expressing WRKY TFs. Overall 68% of the library was revived, with all TF families represented across these wells.

During the Y1H I found six TFs that bound to TaWRKY19 promoter strongly; AtbZIP1, AtbZIP53, AtTCP20, AtHSFB1/4, AtPHL6 and AtATL56. Ideally I would have used a wheat TF library, however, at the time, this was the

easiest option. After performing the experiment I learnt of a Y1H library made up of wheat TFs that are responsive to Septoria infection. Using this library instead would have multiple benefits, with the obvious benefit being that they are wheat TFs. Also, since they were cloned from Septoria infected leaf tissue, I could have assumed they would be more likely to have a role in defence against Septoria[241]. However using an Arabidopsis TF library also had some benefits, for instance the genes were much more likely to have been previously studied, offering insights into their potential function. HSFB1is involved in attenuation of the heat response, repressing heat responsive genes in moderate heat stress[242]. It also controls the balance between growth and defence, regulating the expression of many defence and growth/development genes. Pajerowska-Mukhtar et al[243] showed that knockout mutants could not mount a defence response to the PAMP elf18 (N terminal of the bacterial peptide elongation factor Tu[244]), but could respond to flg22 (N terminal of bacterial peptide flagellin[245]). Ef18 and flg22 trigger different receptors, activating different pathways in response to bacterial infections[243]. As well as responding to direct pathogen challenges, HSFB1/4 also helps prime the plants against future attacks through systemic acquired resistance (SAR)[246]. If the wheat homologue acted in a similar manner it could make a very interesting breeding target, particularly when combined with TaWRKY19, which appears to be a resistance factor.

As previously mentioned nitrogen is very important in wheat growth, with multiple rounds of additional nitrogen (from fertilisers and manure) added by farmers throughout the growth season[247]. Farmers need to apply enough nitrogen for the crop without over applying and therefore wasting money (after reaching yield maximum potential). This makes nitrogen a very interesting area of study for breeding companies. One role of AtTCP20 is in nitrate foraging; with knockout mutant studies showing a need for AtTCP20 in preferential lateral root growth into nitrate rich soil[198]. AtTCP20 is also involved in defence, downregulating expression of LOX2, a jasmonic acid biosynthesis gene[248]. Jasmonic acid (JA) is an important plant hormone with involvement in defence against necrotrophic pathogens (reviewed in [249]). Salicylic acid (SA) is another plant hormone vital to defence. SA is involved in

the defence against biotrophic pathogens and SAR (reviewed in [250,251]). AtTCP20 interacts with AtTCP8, which is a strong inducer of IsoChorismate Synthase 1 (ISC1) expression (involved in SA biosynthesis[252]), implying it may regulate ISC1. The authors theorised the TCP genes may work to regulate the balance between JA and SA mediated defence[253].

AtTCP20 offers an interesting breeding target if the wheat homologue were to act the same. It could potentially increase nitrate foraging efficiency and increase resistance towards biotrophic pathogens, including early Septoria infection. There is anecdotal evidence of a link between high levels of nitrogen and increased infection; hence further studies around AtTCP20 and its wheat homologue may offer an interesting breeding target with the ability to increase nitrogen uptake and decrease biotrophic pathogen infection. One more thing to note about AtTCP20 is its potential expression regulation by SUMO under stress conditions. RNA sequencing performed by fellow laboratory members, Dr. Beatriz Orosa and Dr. Mark Bailey, on SUMO protease knockout plants (Overly Tolerant to Salt 1 and 2 (OTS1/2)) revealed a reduction in AtTCP20 expression after JA treatment when compared to Col-0 plants. PMT control can fine tune responses to stress (reviewed in [254]). The possibility of somehow exogenously inducing SUMOylation events and therefore TCP20 expression when needed is an interesting area for future crop protection.

AtbZIP1 is involved in abiotic stress responses; it is a resistance factor towards salt, drought and osmotic stresses[180]. It is also involved in the regulation of light and nitrogen responses. AtbZIP1 knockouts unusually show an inversion of gene expression in light and nitrogen conditions compared to WT plants[179]. As previously mentioned genes involved in nitrogen responses are of high interest, particularly those that appear to regulate the nitrogen response so drastically as AtbZIP1.

AtbZIP1 and AtbZIP53 are known to act redundantly. They have been implicated together in abiotic stress responses, with mutant studies (knockout and overexpressing) showing an involvement in salt[183] and light stresses[184]. Under salt and light stresses they regulate carbohydrate and amino acid metabolism for plant survival[183,184].

As well as abiotic stress regulation, AtbZIP53 is also involved in developmental control, particularly the promotion of seed maturation[255] and germination[256]. Seed quality is important in wheat crop production, with low quality seeds having a negative effect on yield[257]. If TabZIP98 (AtbZIP53's wheat homologue) acts in a similar way it could make another useful breeding target, offering improved abiotic stress tolerance and seed maturation (faster or increased quality).

AtPHL6 and AtATL56 have not been studied to the same extent therefore I cannot infer any potential role in defence for their wheat homologues due to lack of evidence.

The wheat homologues for the Y1H targets were identified using blast searches. I then ordered primers with the intention of cloning each of the wheat homologues to test their ability to bind to TaWRKY19 promoter. After trying different PCR conditions, cDNAs and polymerases I only managed to successfully clone TabZIP2 (AtbZIP1 homologue). TabZIP2 and AtbZIP1 had similar TaWRKY19 promoter binding strengths (up to 40/60mM, figures 4.4, 4.6 and 4.9).

After some discussion I think I should analyse the Y1H results differently, at the time of the experiment I believed the yeast to have grown strongly based on multiple colonies growing. However these do not necessarily indicate a strong interaction, as only a few cells need to have evolved mutations in either the promoter sequence or have some errant yeast TFs bind to the promoter to cause growth[170]. If there was a true interaction there should be many colonies growing within each of the 5µl spots (as seen with AtbZIP1 in figure 4.6), or a lawn of growth (as seen with AtbZIP53 in figure 4.6). There appears to be some of this growth between AtTCP20 and TaWRKY19 promoter. Another problem with this experiment that I did not consider at the time is that there is very little growth of any of the colonies in –L-W-H media. On reflection I should have also included –L-W media in this experiment to ensure the yeast had mated. Instead I relied on the fact that the protocol[133] included a step in which, post mating, the yeast was incubated in liquid selection media (-L-W). Having this in the figure as well would indicate the level of mating and growth at a baseline level, rather than what I

did at the time, setting the baseline to a condition that already included a selection pressure (-L-W-H).

Another aspect that I did not consider at the time is that with increasing 3AT concentrations the levels of growth should decrease gradually (as in figure 4.6). As is shown in figure 4.3 this is not always the case in my experiments as there are multiple cases in which growth can be seen on higher concentrations of 3AT but not in lower concentrations.

When I repeated these experiments with just AtbZIP1 and AtbZIP53 the results were much more satisfactory. Again I did not include the mated selection pressure, which I would if I were to do it again. AtbZIP53 binds to TaWRKY19 promoter more strongly than AtbZIP1. AtbZIP1 still has some of the errant colonies however there is also a high number of smaller colonies, which indicates a weak binding of AtbZIP1 to TaWRKY19 promoter.

Further experiments are needed to validate these results. Particularly for AtTCP20, AtHSFB1/4, AtPHL6 and AtATL56. Y1H do have problems with high occurrence of false positives[258]. They are useful however as a starting point due to their low cost, speed and ability to screen many different interactions[133,259]. These experiments could include performing ChIP-seq or EMSA. These experiments would confirm the binding of the TF to TaWRKY19 promoter and, with ChIP-seq, also provide the sequences of the other promoters regulated by the TF. This would help to enhance the network surrounding TaWRKY19 and TabZIP2. ChIP-seq has been performed on rice[260,261] and barley. To my knowledge there are currently no examples of ChIP-seq being performed in wheat.

6.3 TabZIP2

Since TabZIP2 was the only wheat homologue I could clone and therefore confirm its ability to bind to TaWRKY19's promoter I focussed on this gene for silencing and infection studies, similar to the studies performed on TaWRKY19.

First an experiment investigating TabZIP2 expression in healthy and Septoira infected wheat was performed. Like TaWRKY19, TabZIP2 is also upregulated

during Septoria infection, with the increase slightly delayed in comparison to TaWRKY19 (10dpi compared to 6dpi respectively). The older healthy samples also have increased expression. It is known that AtbZIP1 (TabZIP2s arabidopsis homologue) is regulated by light, which may have contributed to the increase in expression. However the samples were taken at the same time point each day to mitigate any circadian rhythm effects therefore light should not have an effect in this upregulation.

I proceeded to design and clone two silencing fragments for TabZIP2, leading to successful silencing in wheat. The levels of silencing were similar to that in TaWRKY19 silencing experiments at 40% and 41% for TabZIP2A and TabZIP2B respectively. As mentioned before, I would have liked to investigate the levels of silencing at TabZIP2's highest expression point (10dpi) to get a true assessment of the silencing.

The silenced wheat was then tested in Septoria infection seedling assays. Wheat silenced with TabZIP2 have the opposite phenotype to TaWRKY19 silenced wheat, with the onset of visible symptoms delayed compared to the BSMV:00 control wheat (figure 4.16). The Septoria infection was impaired, with a reduction in spore production on TabZIP2 silenced wheat (figure 4.18). These results indicate that TabZIP2 is a negative regulator of wheat defence against Septoria, opposite to TaWKRY19.

Many papers have used infection symptoms, pycnidia counts and spore counts as an indication for Septoria's infection ability[26,119,120,262] with more reliance on spore counts and infection symptoms rather than pycnidia counts[26,50,230]. Infection symptoms can either be scored by assessing the onset of visible symptoms or by assessing the percentage of leaf covered with necrosis and pycnidia at a certain time point after infection. The second method takes into account pycnidia but does not count them directly, instead setting a minimum pycnidia concentration for when the area should be counted as diseased[45].

Further experiments using qRT-PCR to measure fungal biomass (after DNA extraction from infected wheat) can also be performed to enhance results. I did begin to work on performing these experiments but did not complete this.

Ideally I would have tested the TabZIP2 knockdown plants ability to defend against abiotic stresses to see if it functioned the same as AtbZIP1. Unfortunately I did not have time to do this and abiotic stress was not the main focus of my project. Abiotic stresses cause major crop losses[263,264] and since AtbZIP1 positively defends against abiotic stresses[180] there is a possibility that TabZIP2 could also be a positive regulator against abiotic stress. This would not be ideal with my results indicating a negative regulation against Septoria infection.

After showing TabZIP2 binds to TaWRKY19s promoter (via the Y1H), the next step was to see whether TabZIP2 controls TaWRKY19 expression positively or negatively. To do this I used TabZIP2 silenced wheat samples to measure the expression of TaWRKY19. The results of this indicate that TabZIP2 may be a negative regulator of TaWRKY19 (figure 4.22). Through the Y1H experiment I found at least 5 other TFs that potentially regulate TaWRKY19s expression. This can be seen through TaWRKY19 and TabZIP2's expression profiles in healthy and Septoria infected wheat. TabZIP2 expression sharply rises at 10dpi (figure 4.11), but this does not cause a decrease in TaWRKY19 expression (figure 3.2), in fact TaWRKY19's highest expression point is at 12dpi. Further confirmation of other TFs regulating TaWRKY19 expression comes from investigating the expression of both genes in different wheat varieties (figures 4.20 and 4.21). There appears to be no correlation between the expression levels of TaWRKY19 and TabZIP2 across the wheat varieties. This experiment was performed on seedlings (4-weeks-old); older leaves (such as the flag leaf) may show a different correlation.

Although TabZIP2 was one of the weaker interactors from the TaWRKY19 promoter binders list it is known that bZIP TFs are regulated by phosphorylation[265]. My research group is highly focussed upon PTMs, another reason for focussing on AtbZIP1. Other studies have postulated to the ability of PTMs to change binding capacity of AtbZIP1[181]. Ideally I would have liked to investigate whether mutations in the phosphorylation site change the binding strength of AtbZIP1 and TabZIP2 towards TaWRKY19 promoter. Phosphorylation is important in defence, the MAPK cascade is

activated once a pathogen is perceived (reviewed in [35,36]). Activation of this pathway leads to successive rounds of phosphorylation, the outcome of which may be the potential phosphorylation of TabZIP2. My experiments indicate that TabZIP2 is actually a negative regulator of defence and TaWRKY19 (which is a positive regulator of defence). Therefore it may be activated (potentially through phosphorylation) to ensure a defence response is not activated prematurely, as it is an energy costly process[266].
As previously mentioned, AtbZIP1 regulates nitrogen and light responses. If the same is true for TabZIP2 it may offer an interesting link between the nitrogen and defence paradigm[267-269]. As previously mentioned, Dr. Jack Lee performed qRT-PCR experiments studying expression changes of TabZIP2 after watering with different nitrogen concentrations. Unfortunately there did not appear to be any difference between the three nitrogen concentrations applied and TabZIP2's expression. Interestingly, TaWRKY19s expression was upregulated in low nitrogen concentrations; potentially showing TaWRKY19 is regulated by nitrogen. This result is in line with the high nitrogen = high infection concept as TaWRKY19 is a positive regulator of defence; therefore as nitrogen is increased, TaWRKY19 expression is decreased leading to increased susceptibility. These experiments were performed on 4-week-old seedlings, thus do not necessarily translate to the field. A large field trial was performed by KWS during this project. In this trial two different nitrogen fertiliser concentrations as well as two fungicides, one against everything but Septoria and the other also controlling Septoria, were applied to 8 different wheat varieties. As of writing, flag leaf samples from this trial were being processed ready for use in qRT-PCR to measure the expression of certain genes, including TaWRKY19, TaWRKY9 and TabZIP2. This could confirm TaWRKY19s nitrogen regulation and may show a link between TabZIP2, nitrogen (as with AtbZIP1) and defence.
It is possible to silence two genes at the same time using VIGS[270]. Since TabZIP2 appears to be a negative regulator of TaWRKY19 and they have opposing defence roles it would be interesting to see if silencing both concurrently led to a WT phenotype or whether one of the genes has a larger effect.

Interestingly, AtbZIP1 (and therefore maybe TabZIP2) is negatively regulated by sugar[182,271,272]. With TaWRKY19 potentially increasing sugar content[152] there may be a feedback loop towards TabZIP2. As TaWRKY19 positively regulates sugar levels, defence increases and TabZIP2 translation decreases, which is potentially a negative regulator of TaWRKY19 leading to an increase in TaWRKY19 expression (figure 4.22).

I also investigated whether TabZIP2 regulates other TaWRKY genes. I chose two newly identified WRKY genes; TaWRKY41 and TaWRKY63. These genes were identified by Sarris et al[273] who searched multiple plant genomes for the presence of NBS-LRR-fusion proteins. NBS-LRR proteins are important in pathogen perception[273-275]. TabZIP2 only appears to regulate TaWRKY63 (negative regulation) but not TaWRKY41 (figures 4.23 and 4.24). This is interesting as the assumption is that these genes work at early defence detection. Therefore TabZIP2 is potentially redirecting resources towards late defence or growth after its upregulation post pathogen perception. I did perform initial silencing and infection experiments on both TaWRKY41 and TaWRKY63 but they were unsuccessful. This was disappointing as it would be useful to find breeding targets which focuses on early perception, potentially stopping Septoria infection (by increased plant perception) before it has a chance to switch to necrotrophic growth and cause damage.

The final thing I focussed on in chapter 4 was the potential for a feedback loop between TaWRKY19 and the other Y1H targets TaHSFB1 and TaTCP20. Similar to TabZIP2, TaTCP20 does not appear to be regulated by TaWRKY19. TaHSFB1 however does appear to be regulated by TaWRKY19 (figure 4.27), becoming upregulated in both TaWRKY19 silenced lines. Feedback loops are important as they allow a rapid increase in the response, or they can dampen the response to save energy[266].

6.4 TaWRKY9

The other WRKY gene identified as a candidate in the initial experiments was TaWRKY9. Unfortunately due very low/zero expression levels of TaWRKY9 in the healthy samples I did not show the qRT-PCR results of TaWRKY9 expression in healthy and Septoria infected time courses. On reflection I

would have repeated these experiments with increased cDNA in the qRT-PCR for both healthy and infected wheat to ensure a signal is detected. Initial experiments indicated TaWRKY9 was upregulated in infected tissue, this was partially confirmed in the qRT-PCR since only the infected tissue samples were detected past the threshold value.

I still decided to perform silencing and Septoria infection experiments upon TaWRKY9, even without solid evidence of expression changes. In order to have a silencing fragment with low levels of off target silencing I performed RACE PCR experiments to sequence the UTRs of TaWRKY9. The 3' UTR for TaWRKY9 was sequenced, with one silencing fragment designed against it due to its small size (132bp)(figure 5.4). UTRs are important in gene regulation, with 18-19% of all transcribed DNA in Arabidopsis and rice encoding for UTRs[276]. A recent review by Srivastava et al[276] highlights the importance of UTRs in gene regulation in plants. They would like to see more studies focussing on both the CDS and the UTR, particularly in crop plants. After designing the silencing fragment against the 3' UTR I then blasted it to test for any potential off target silencing. The blast was performed against cv. Chinese Spring, whereas I identified the 3' UTR sequence from cv. Avalon. Interestingly there is a SNP located in the UTR, potentially causing a difference in regulation of TaWRKY9 through the UTR in the two varieties. Both varieties may show similar expression levels, but have different protein levels due to this regulation. Silencing TaWRKY9 in different varieties and infecting them with Septoria whilst also sequencing the full coding region may give insights to a link between UTRs and defence.

Silencing of TaWRKY9 was successful, with an average decrease in expression of 46% across the three experiments (figure 5.7). These plants were then used in Septoria infection experiments. As with TaWRKY19, if I were to repeat the experiments again I would have measured silencing levels in Septoria infected leaf tissue, as TaWRKY9 is upregulated after infection. Septoria infection on TaWRKY9 silenced plants is impaired, with a delay in the onset of necrotrophic growth (figure 5.8), reduced pycnidia (35%, figure 5.9) and spore (51%, figure 5.10) production. These results indicate that TaWRKY9 is a negative regulator of defence.

After determining a role in defence for TaWRKY9 the next step was to investigate upstream genes in the hope of finding another TF that could have a larger effect in the defence response pathway. It also gives an idea of the function of TaWRKY9. A 1,973bp region upstream of TaWRKY9s ATG was identified and cloned (figure 5.11) ready for experimentation through a Y1H screen. The same library was used as with TaWRKY19. Through the initial screen 62 TFs were identified (table 5.3). This was later reduced to eight TFs after increased 3AT selection pressure was applied – AtRMR1, AtbHLH010, IIF factor beta subunit, AtSCL26, AtDREB19, AtMYB8, AtAGL3 and AtAGL73 (figure 5.12).

Interestingly of the top eight TaWRKY9 promoter binders, AGL3, AGL73, bHLH10 are all involved in floral development[213,215,277,278] or fertility[277]. Also DREB19 is more expressed in seedling and inflorescence tissue with overexpressers flowering sooner than WT plants[279]. The Y1H experiments did not indicate whether the TFs positively or negatively regulated TaWRKY9 expression. Since it is a negative regulator of defence and TFs involved in development bind to its promoter, TaWRKY9 may be involved in the balance between growth and defence. I did perform initial experiments testing the growth phenotypes of TaWRKY9 knockdown plants. There were some differences, with a slight decrease in leaf length compared to BSMV:00 treated control plants. However there were no differences in the number of tillers and I did not measure yield due to time limitations and the small size of the experiment. Therefore field experiments looking into the growth, flowering, flowering time and yield of TaWRKY9 knockdown, TILLING or transgenic knockout lines would be interesting.

DREB19 expression is highly upregulated in salt, drought and heat stress, with overexpressers having enhanced tolerance to salt and drought stress[279]. Drought and salt stress are important abiotic stresses in crop production, leading to a potential 50% reduction in yield[263,264]. Thus making it another important focus for plant breeders, particularly if there is a link to defence responses through TaWRKY9.

DREB19 is not responsive to the plant hormones SA and JA, which are important in plant defence. Initial experiments investigating the potential for

a feedback loop indicate that TaWRKY9 is a negative regulator of AtDREB19's wheat homologue (figure 5.14). In TaWRKY9 knockdown plants, TaDREB19 expression is increased almost 7 fold. Further repeats of this experiment are needed to validate this result. However of all the real time experiments performed in this thesis it does represent the highest average change in expression. Experiments to ensure the wheat homologue also binds to TaWRKY9 are also needed before more in depth studies into the interactions and function of TaDREB19 are to be performed. Experiments could be performed testing the ability of TaWRKY19 silenced plants to deal with abiotic stresses such as salt and drought (as overexpressing AtDREB19 plants are resistant), which may be due to the increased expression of TaDREB19. Simultaneously silencing both genes, as well as each individually, would indicate which gene is dominant, with the Y1H experiments indicating AtDREB19 binds to TaWRKY19's promoter but then TaWKRY19 silenced plants indicating that it has a role in negatively regulating TaDREB19.
After looking into the function of AtRMR1, I am not convinced that outside of yeast it truly binds to TaWRKY9 promoter. Although other RING-H2 domain proteins are known to regulate transcription[280,281] (hence why it was included in the Y1H library), I do not believe this particular gene does. This is based on the fact that it contains a transmembrane domain and an N terminal protease-associated domain that interacts with sequence specific vacuolar sorting determinants[282,283]. There is strong evidence of its cargo receptor function, trafficking towards the protein storage vacuole[284,285]. The C terminal domain is cytosolic facing and containing a RING-H2 domain, the function of which is unknown. Also it does not appear to localise to the nucleus, it is instead localised to the endoplasmic reticulum[282,286,287] and *trans*-golgi network (when heterodimerised with AtRMR2)[287]. Therefore it is not surprising that the wheat homologues expression does not change in TaWRKY9 knockdowns.

6.5 Conclusion

With the ever-changing environment, increase in population and decrease in farming land there is a need to ensure our crops are more productive in a

smaller space. Septoria can cause up to 50% losses in wheat yield[3]. Efforts to control Septoria are made more difficult by its stealthy growth and rapid resistance evolution[3,14,17,24]. TFs are ideal candidates for breeding targets[55]. They have the ability to control multiple genes and lead adaptation against stresses[288]. WRKY and bZIP TFs were found to show high levels of expressional change post Septoria infection, they have also been shown to be involved in plant defence previously. Throughout this project I have identified two WRKY TFs and one bZIP TF that have a role in defence against Septoria. Further field experiments, potentially using TILLING lines, are needed to validate my results. However my hope is that these can be used as breeding targets for the generation of new Septoria resistant varieties. The two WRKY genes have already been patented (in collaboration with KWS) for this very function.

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8. Supplemental

Supplemental table 1

Primer name	Sequence	Melting temp	Notes
AtBZIP53 protein F	CACCATGGGGTCGTTGCAAATGCA	55	Cloning protein
AtBZIP53 protein R	TCAGCAATCAAACATATCAGCAGAAGC	55	Cloning protein
BSMV seq F	GGTGCTTGATGCTTTGGATAAGG	55	Sequencing
BSMV seq R	TGGTCTTCCCTTGGGGGAC	55	Sequencing
M13 R	CAGGAAACAGCTATGAC		Sequencing
pJET1.2 R	CGACTCACTATAGGGAGAGCGGC		Sequencing
RT CDC48 F	GTCCTCCTGGCTGTGGTAAAAC	60	real time
RT CDC48 R	AGCAGCTCAGGTCCCTTGATAC	60	real time
RT EF1a F	A CCTGAAGAAG GTCGGCTACA A	60	real time
RT Ef1a R	ATCTGGTCAAGCGCCTCAAG	60	real time
RT HSFB1/4 F	GCACTGCAACTTCTCCTCCT	60	real time
RT HSFB1/4 R	GGTTTTGGAGGACTGGGGAG	60	real time
RT TabZIP2 F	CTACACGACCGAGCTGACC	60	real time
RT TabZIP2 R	TCAGAACTGGAACGCGTCA	60	real time
RT TaDREB19 F	TCCACGACAATCCCAACCTC	60	real time
RT TaDREB19 R	CGGGTACTTTCCTCTGCTCC	60	real time
RT TaRMR1 F	GGAACTGCCGACTTTTACGC	60	real time
RT TaRMR1 R	CCACTTTGTCAGCCAGGGAT	60	real time
RT TaTCP20 F	AGAATTCCCTCCGGTTCAGG	60	real time

RT TaTCP20 R	GGGAGGATAAAATTGCCAGCC	60	real time
RT TaWRKY19 F	GTGATATCCGGGAAGGTGATGGTG	60	real time
RT TaWRKY19 R	GCTCCTTGGGTGAGGATTTCCTCTC	60	real time
RT TaWRKY41 F	CGGCTCCAGGAACCCAAGAG	60	real time
RT TaWRKY63 F	CCCACCTCCAGCATACAGTGT	60	real time
RT TaWRKY63 R	TCCTATGATTGTTGCTGCTGGA	60	real time
RT TaWRKY9 F	ACGGGGCTACTACAAGTGCA	60	real time
RT TaWRKY9 R	GGCGGACACACTAGCTACGG	60	real time
RT TaWWRKY41 R	TCTGCTCATACCCAGGCTGTG	60	real time
TabZIP2 fragA F	AAGGAAGTTTAAGAGAGCAACATCGACGGCG	55	Cloning silencing fragment
TaBZIP2 fragA R	AACCACCACCACCGTTCAGCTCGGTCGTGTAGGCG	55	Cloning silencing fragment
TabZIP2 fragB F	AAGGAAGTTTAACAAAGTGGACGGCGAGAACG	55	Cloning silencing fragment
TabZIP2 fragB R	AACCACCACCACCGTTCAGAACTGGAACGCGTCAG	55	Cloning silencing fragment
TabZIP2 protein F	GGCGACGACATGTTTTTTTGTTC	55	Cloning protein
TaBZIP2 protein R	GACTCTTAAGGAACTGGAACGCGTCAGG	55	Cloning protein
TaWRKY19 fragA F	AAGGAAGTTTAATTGGTCCCCGTGTCTTCTTT	55	Cloning silencing fragment
TaWRKY19 fragA R	AACCACCACCACCGTGAGCAATAACAGCCCGTCAC	55	Cloning silencing fragment
TaWRKY19 fragB F	AAGGAAGTTTAATGCTCTGTTTGCTGTATATTCCC	55	Cloning silencing fragment
TaWRKY19 fragB R	AACCACCACCACCGTGACCACCCATCATGTTCAACA	55	Cloning silencing fragment
TaWRKY19 prom	CACCAGGGCGTATTTCCTTCAGCG	55	Cloning promoter

F			
TaWRKY19 prom R	GGAGGGAGGGATGCTTTCTG	55	Cloning promoter
TaWRKY19 protein F	CACCATGGCGGCGGGGCAGTGGTCA	touchdown	Cloning protein
TaWRKY19 protein R	CTAGAAGGCGAGATCGTTCAGAATGGCTG	touchdown	Cloning protein
TaWRKY19 RACE 3' 1	CGTGAGTACAACGAGCGATGC	50	RACE PCR
TaWRKY19 RACE 3' 2	GGCGCAAGTATGGACAGAAAGTGGTG	50	RACE PCR
TaWRKY19 RACE 3' 3	CGTGCCTGAGTCTAGGAACAGAAGCC	50	RACE PCR
TaWRKY19 RACE 5' 1	TCCCGCAACTGTTTGTTTTGAGCC	50	RACE PCR
TaWRKY19 RACE 5' 2	AGTTGTCCTGGGGGTACCCGAGGAG	50	RACE PCR
TaWRKY19 RACE 5' 3	TCCACCGAAGAAGCCCAGCGCCGA	50	RACE PCR
TaWRKY9 fragA F	AAGGAAGTTTAA	55	Cloning silencing fragment
TaWRKY9 fragA R	AACCACCACCACCGT	55	Cloning silencing fragment
TaWRKY9 prom F	CACCCGAAGCTCTGGTGTTGATTCC	55	Cloning promoter
TaWRKY9 prom R	CTGTGGCTCTGCAGATCTTG	55	Cloning promoter
TaWRKY9 RACE 3' 1	GTGACGTCCACGTCCTTCTTTCTC	50	RACE PCR
TaWRKY9 RACE 3' 2	ACCCGGCGATGCTGGTGGTGACG	50	RACE PCR
TaWRKY9 RACE 3' 3	GCCGTCGCCGGTGCCGATTCCGA	50	RACE PCR
TaWRKY9 RACE	TCAGGCTCTGCGGCTGCGGGAGA	50	RACE PCR

5' 1			
TaWRKY9 RACE 5' 2	GCGATCTCGCCGAACGGCTGCTGCA	50	RACE PCR
TaWRKY9 RACE 5' 3	GCCGGTGTGAGGTCGCCTGTGA	50	RACE PCR
YFP F	GGTCCTTCTTGAGTTTGTAAC		Sequencing

List of primers used in this thesis, including name, sequence, melting temperature used in PCR and notes for their use.

Supplemental table 2

gene name	healthy vs infected
TraesCS3D01G001500	-12.46796895
TraesCS1A01G165500	-12.30460853
TraesCS5D01G032000	-11.80401412
TraesCS1D01G083100	-11.55048605
TraesCS2B01G242600	-11.37662775
TraesCS6B01G185000	-10.98053103
TraesCS4B01G091700	-10.84325901
TraesCS1D01G444600	-10.78844863
TraesCS1D01G040300	-10.72691213
TraesCS2B01G608800	-10.65981133
TraesCS7A01G053400	-10.65364691
TraesCS4D01G073700	-10.55872105
TraesCS1B01G367500	-10.55781121
TraesCS3B01G457000	-10.55446345
TraesCS2D01G026900	-10.51948996
TraesCS3D01G513800	-10.49518889
TraesCS2A01G420200	-10.46589682
TraesCS4A01G037500	-10.45461576
TraesCSU01G160400	-10.44675985
TraesCS2B01G020200	-10.40948745
TraesCS4D01G154700	-10.39350611
TraesCS5A01G503300	-10.33523956
TraesCS3A01G019400	-10.3211465
TraesCS6B01G441800	-10.30982594
TraesCS3D01G519600	-10.16062639
TraesCS6B01G017500	-10.14765296
TraesCS2B01G416000	-10.12267768
TraesCS1A01G132000	-10.08648112
TraesCS7D01G159100	-10.00823973
TraesCS1B01G444600	-9.975884608
TraesCS3B01G320000	-9.928858121
TraesCS3B01G510100	-9.89428376
TraesCS5D01G120600	-9.858715391
TraesCS1A01G114600	-9.833694213
TraesCS7B01G449900	-9.832497425
TraesCS1B01G442700	-9.767733787
TraesCS2D01G573600	-9.746187686

TraesCS5D01G528100	-9.742050805
TraesCS3D01G010300	-9.733271849
TraesCS2A01G586000	-9.66083441
TraesCS5A01G116200	-9.564752448
TraesCS3B01G456000	-9.547019314
TraesCS6A01G052200	-9.517701149
TraesCSU01G084200	-9.451207618
TraesCS3D01G513600	-9.404360085
TraesCS6A01G015300	-9.398753573
TraesCS1B01G450000	-9.394549961
TraesCS6D01G034900	-9.392763847
TraesCS1A01G189500	-9.372629915
TraesCS3A01G375500	-9.370443785
TraesCS3B01G366400	-9.338199319
TraesCS1D01G044300	-9.299925968
TraesCS7A01G002300	-9.287906542
TraesCS7A01G425700	-9.285879865
TraesCS6D01G005800	-9.2505428
TraesCS3A01G401800	-9.226802334
TraesCSU01G110100	-9.223413541
TraesCS7A01G002500	-9.221368642
TraesCS2D01G142200	-9.219658347
TraesCS4D01G186400	-9.19926061
TraesCS1B01G053200	-9.197182227
TraesCS5D01G032100	-9.191324005
TraesCS3B01G273100	-9.182310245
TraesCS6B01G238800	-9.176544052
TraesCS2D01G573800	-9.160393152
TraesCS7A01G432600	-9.150957787
TraesCS6A01G305000	-9.120630335
TraesCS6B01G442200	-9.076482762
TraesCS7B01G493000	-9.076316597
TraesCS5A01G412500	-9.047494591
TraesCS4A01G037600	-9.016064961
TraesCS2D01G397400	-8.981945815
TraesCS2A01G124200	-8.973863566
TraesCS2A01G024200	-8.972223037
TraesCS2D01G548100	-8.968500611
TraesCS6B01G370000	-8.964026247
TraesCS2A01G465300	-8.955040088
TraesCS5A01G470500	-8.925632194
TraesCS2A01G554400	-8.8870697
TraesCS6D01G364800	-8.871366582
TraesCS5B01G547300	-8.860450629
TraesCS5D01G067100	-8.856059626
TraesCSU01G084300	-8.820206635
TraesCS1B01G305800	-8.808966263
TraesCS1A01G051200	-8.80152768
TraesCS2B01G555700	-8.786580425
TraesCS5B01G044800	-8.764159303
TraesCS6B01G129000	-8.764153726
TraesCS7B01G114600	-8.761670369
TraesCS4A01G419100	-8.759778335
TraesCS5D01G525000	-8.759488644
TraesCS1D01G040500	-8.740595736
TraesCS2A01G333300	-8.725775963
TraesCS6B01G371700	-8.70421942
TraesCS3B01G056400	-8.696128154
TraesCS2D01G284700	-8.679663409
TraesCS3D01G006700	-8.678388791

TraesCS2B01G357200	-8.676386645
TraesCS2D01G305300	-8.672602674
TraesCS6B01G228400	-8.668605467
TraesCS2B01G168500	-8.181571132
TraesCS7A01G110100	-7.773402488
TraesCSU01G063100	-7.742510872
TraesCS3B01G014800	-7.495728664
TraesCS1B01G352400	-7.290089155
TraesCS3D01G047200	-7.181430849
TraesCS6D01G386500	-6.966704954
TraesCS1A01G222500	-6.92479527
TraesCS3B01G496100	-6.838328334
TraesCS1B01G157200	-6.820333314
TraesCS4B01G177800	-6.685419808
TraesCS1D01G044400	-6.587624469
TraesCS1B01G115700	-6.429718174
TraesCS4B01G316400	-6.400746092
TraesCS1A01G136600	-6.399746789
TraesCS7D01G544300	-6.384976999
TraesCS2D01G230000	-6.374098618
TraesCS5B01G390000	-6.217342724
TraesCS1D01G238800	-6.189090837
TraesCS7B01G005900	-6.175748428
TraesCS7B01G296600	-6.174631799
TraesCS3B01G478400	-6.174086691
TraesCS5D01G068300	-6.174040397
TraesCS7A01G083500	-6.159507584
TraesCS6A01G115700	-5.992989707
TraesCS7B01G253600	-5.971703418
TraesCS2A01G371300	-5.959079564
TraesCS4B01G113500	-5.944581385
TraesCS7A01G359700	-5.944168627
TraesCS2B01G085600	-5.901141926
TraesCS5B01G357000	-5.747534703
TraesCS5B01G518100	-5.70426809
TraesCS7A01G089400	-5.686603071
TraesCS3A01G407900	-5.686400888
TraesCS3A01G363300	-5.685866852
TraesCS5A01G391500	-5.642697143
TraesCS2A01G345500	-5.540905635
TraesCS5A01G534100	-5.374116709
TraesCS6D01G366600	-5.373972881
TraesCS1B01G018200	-5.348543348
TraesCS5D01G525300	-5.015322539
TraesCS6A01G076500	-5.011666404
TraesCS2D01G371700	-5.005430909
TraesCS4B01G064600	-4.974568504
TraesCS1D01G293600	-4.973001847
TraesCS4D01G134900	-4.679869708
TraesCS6D01G339100	-4.636675338
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TraesCS7A01G005900	2.387412857
TraesCS6A01G375100	2.395042467
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TraesCS7D01G421100	10.62617875
TraesCS6A01G179100	10.62838483
TraesCS5B01G274100	10.76930721
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TraesCS1A01G009800	10.85711554
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TraesCS5D01G398800	10.91061219
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TraesCS4B01G005900	11.85502124
TraesCS1B01G076800	11.86599833
TraesCS3B01G478000	11.87221174
TraesCS2B01G034700	11.9291526
TraesCS2A01G026700	11.99966499
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TraesCS1B01G450300	12.23204774
TraesCS3B01G011300	12.25089768
TraesCS2A01G312400	12.4701913
TraesCS4B01G332300	12.49235669

TraesCS2B01G121700	12.50980144
TraesCS2B01G038700	12.52609103
TraesCSU01G242100	12.57252024
TraesCS3A01G444100	12.60456546
TraesCS2B01G240300	13.23030818
TraesCSU01G159400	13.5212329
TraesCS6D01G169600	13.89693787
TraesCS6B01G035500	13.99571664
TraesCS7B01G440100	14.2038186
TraesCS2B01G038600	20.15106981
TraesCS5B01G277800	20.8386173

Table to show the differential expression and gene numbers between healthy vs Septoria infected samples from an RNA sequencing experiment. Samples include both BSMV:00 and BSMV:TaWRKY19B silenced wheat.

Supplemental table 3

gene name	BSMV:TaWRKY19 vs BSMV:00
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TraesCS7B01G354900	-6.331406733
TraesCS2D01G023100	-6.328473659
TraesCS2B01G482800	-6.313016841
TraesCS2B01G627600	-6.300502952
TraesCS2B01G483600	-6.294465814
TraesCS5D01G448800	-6.052748251
TraesCS3D01G030700	-6.023498465
TraesCS1D01G413400	-6.011520028
TraesCS4B01G237500	-6.01093553
TraesCS4B01G023700	-6.000494218
TraesCS2D01G421700	-5.982731621
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TraesCS4B01G281800	-5.883745644
TraesCS2B01G038600	-5.671295459
TraesCS5B01G277800	-5.669891762
TraesCS6A01G122600	-5.632102907
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TraesCS6B01G207600	-5.626143887
TraesCS1D01G181100	-5.62496194
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TraesCS6A01G115200	-5.595273216
TraesCS5A01G333900	-5.594604886
TraesCS2A01G057000	-5.580113671
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TraesCS7A01G171600	-5.571295362
TraesCS3B01G493900	-5.558621111
TraesCS7B01G384300	-5.549225273
TraesCS5D01G120800	-5.546986561
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TraesCS5B01G365500	-5.543630709
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TraesCS7D01G517700	-5.534502542

TraesCS4A01G021700	-5.079217297
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TraesCS5A01G094700	-5.046558198
TraesCS2B01G536400	-5.046409762
TraesCS3D01G450100	-5.045201208
TraesCS6B01G147900	-5.039826895
TraesCS1D01G426300	-5.035613408
TraesCS4A01G234200	-5.035520779
TraesCS5B01G139200	-5.032591436
TraesCS1A01G377300	-5.032199209
TraesCSU01G209300	-5.010779769
TraesCS7D01G019200	-5.007480246
TraesCS7A01G089300	-5.000066898
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TraesCS1A01G362900	8.197807048
TraesCSU01G094800	8.60757961
TraesCS2D01G264400	8.666056769
TraesCS7A01G373300	8.691582604
TraesCS1B01G056900	9.179916431
TraesCS1D01G044300	19.7863091

Table to show the differential expression and gene numbers between BSMV:TaWRKY19B vs BSMV:00 samples from an RNA sequencing experiment. Samples include both healthy and Septoria infected (12dpi).

Supplemental table 4

Family	No. of TFs	Hit Sequence
AP2;ERF	62	AACTA, aggCGGCGg, agGCGGCggg, aggCGGCGgg, ATCAA, ATCCA, ATCGA, ATCTC, ATCTG, atgGCGGCgc, atggCGGCGc, CAGAT, catggCGGCG, catgGCGGCg, cCGCCGgc, cCGCCGgc, ccGCCGGcat, ccgCCGGCat, ccgGCGGCga, ccgGCGGCtc, cgaggCGGCG, cgCCGACa, cgcCGACAca, cgCCGACaca, cgCCGACg, cgCCGACgcg, cgCCGGCa, cGCCGGca, cgCCGGCt, cGCCGGct, cgCGCCGaca, cgGCCGGcgg, cggCCGGCgg, cGGCGGcg, cggCGGCG, cgGCGGCgaa, cggCGGCGaa, cGGCGGctc, cgGCGGCtcc, ctCCGACg, CTCTA, GAGAT, gaggCGGCGg, gagGCGGCgg, gCCGAC, gcCGGCGa, gcCGGCGg, gcgCCGACa, gCGCCGaca, gcgCCGACac, gCGCCGacac, gCGCCGgc, gcGCCGGcga, gcgCCGGCga, gctCCGACga, gGCCGGcg, ggCCGGCg, ggCGCCGgcg, gGCGGCga, ggCGGCGc, ggGTCGGcg, ggGTCGGcgc, gGTCGGcg, gGTCGGcgc, ggtCGGCGc, ggtCGGCGcg, GTCGGc, GTCTA, TAAAT, TAAGAgcaact, TAGAA, tCCGAC, tcCGCCGgca, tccggCGGCG, tccgGCGGCg, tcgCCGACg, tcgCCGACgc, TGGAT, tggCCGCCg, tgGCCGCcgg ,tggCGGCG, tgGCGGCg, tGGCGGcgc, tggCGGCGc, tgGCGGCgcc, tggCGGCGcc, tggGTCGGcg
bZIP	30	aaCACGTatg, ACACCtgc, ACACCtgg, ACACGcgc, ACACGtat, acaCGTAT, aCACGTat, acgaaCACGTatg, acGACGTggc, acgACGTGgc, acgACGTGgca, acgACGTGgcacg, ACGTA, ACGTG, AGACG, aGCGGT, ATGACatgat, CACGT, CATCA, CCACGcga, CCACGcgatc, CCACGcgc, CCACGcgctc, CCGAGcgtcccacttg, CCTCA, CGACA, CGACG, cgACGTGg, cgaCGTGG, cgaCGTGGc, cgaCGTGGca, cgACGTGgca, CGGCA, cggCGTCAcg, cggCGTCAcga, CGTAA, CGTCA, CGTCC, CGTCG, CGTCT, CGTGA, CGTTA, CTTCA, gacgACGTGg, gacgaCGTGG, gacgACGTGgca, gacGACGTggca, gacgACGTGgcacgagcca, GACGT, gCCACGcga, ggacGACGTggc, ggCCACGcgatc, TCACG, TGACA ,tGACAGcaga, TGAGG, TGATG, TGCCG, TGGCG, TGTCG, TTACG
Dof	18	aAAAAGc, aAAAAGt, aAAAAGttt, AAAGA, AAAGC, AAAGCa, AAAGG, AAAGT, AAGGA, AAGGC, AAGGG, ACCTT, ACTTT, caAAAAGctc, caAAAGAgga, caAAAGAggc, cacAAAAGagg, ccaaAAAGCtc, CCCTT, CCTTT, cgaAAAAGt ,gaAAAAG, gaAAAAGt, gaAAAAGttt, gacAAAAGagg, gaggAAAGCtc, GCCTT, TCCTT
NAC;NAM	16	aaCACGCaag, aacACGTAtg, aCACGCaa, aCACGCaag ,acaCGCAAgc, aCACGCaagc, acaCGCAAgca, caacACGCAa, caaCACGCaa, cACATG, cCACGCgatc
TCP	11	aATAAAta, ACCCG, ACCCGc, ACCCGg, AGCCC, AGGCC, cCGGGT, CGACC, CGCCC, CGGCC, CGGGC, CGGGT, CGTCC, CGTCCcac, cgtgCCCACa, ctGGACCagc, gaaGGACC, GAGCC, GCACC, GCCCC, GCGCC, GCTCC, GCTCCgac, GGAAC, GGACA, GGACC, GGACG, GGACT, GGAGC, GGATC, GGCAC, GGCCA, GGCCC, GGCCG, GGCCT, GGCGC, ggcGGCCC, GGCTC, GGGAC, GGGCC, GGGCG, GGGCT, GGGGC, gggGGACG, GGGTC, GGTCC, GGTCCcgg, GGTCG, GGTCT, GGTGC, GGTTC, GTCCC, GTGCC ,gtgCCCAC, gtgCCCACaa, GTTCC, TGCCC, tGGACCag, TGGCC, TGTCC
AP2;B3;RAV	10	aacACCTGcaca, ctgCAACAcaaa, gacACCTGgcgc ,gCAACAca, ggtcTGTTGgtt, tccACCTGgggt, tcTGTTGg, tcTGTTGgtt, ttTGTTGc, ttttTGTTGcct
AP2;RAV;B3	10	CAACA, CACCTg, TGTTG
bHLH	9	acaCCTGC, ACACGcgc, acaCGCGC, ACACGtat, acaCGTAT, ATACTtga, ATCCGggt, CAAATg, cAAATG, CACATg, cACATG, CACGAg, cACGAG, CACGCg, cACGCG, CACGTa, cACGTA, CACTTg, cACTTG, CAGTTg, cAGTTG, CATTTg, cATTTG, CCACGcgc, ccaCGCGC, cccACTTGg, GACGTg, gACGTG, gACGTGg, GCACAtga, GCACGagc, gcaCGAGC, gccACTTGg, GCGCGtac ,gcgCGTAC, ggaCCTGC, GGACGggc, ggaCGGGC, ggcCGTGC, ttggacgACGTGgcacg, ttggacgACGTGgcacg
GATA;tify	9	AATCA, AATCT, AGATA, AGATG, ATATC, atgGATCTga, CATCA, CATCG, CGATC, CGATG, CGATT, CTATC, GATAA, GATAT, GATCC, GATCG, GATCG, GATCT, gatgGATCTg, GGATC, GGATC, GGATG, GGATT, GTATC ,TATCA, TATCC, TATCT, TGATA, TGATC, TGATG, tgGATCTga
AT-Hook	8	aAAATAc, aAAATAt ,acAAATAtct, ATTGCaaaac, ATTTTtg, gggaATAAA, ggggAATAAa, TTTTTccttc, TTTTTccttc
Others	8	ACCGAga, cAACCG, cAACGG, CACGTa, cACGTA, CAGTTa, cAGTTA, CAGTTg, CCGTTa, CCGTTg, CGGTTa, cgTTACA, CTGTTg, GACGAc, gACGAC, GACGGc, gACGGC, GACGTg, gACGTG, GCCGAca, GGCGTc, gGCGTC, tAACCG ,TCCACctggg, tgCCGGT, TGCGTa, tGCGTA, tgTCCGT, tgTTGGT, TTACCatgtttagaag
Homeodomain;HD-ZIP	6	caAATCAtgt, cAATCAttca, caccAATCAt, ccAATCAt, ccAATCAttc
B3	5	AATGC, ACATG, agCATGCtc, aGCATGctc, atGCATGa, CAAGC, CACGC, CAGGC, cagtTGTAGc, CATCC, CATGA, CATGC, CATGG, CATGT, CATTC, CCATG, CCTGC, CGTGC, CTTGC, GAATG, gaGCATGct, gagCATGCt, GATGC, GCAAG, GCACG, GCAGG, GCATA, GCATC ,GCATG, GCATT, GCTTG, GGATG, GTATG, TATGC, TCATG
Homeodomain;TALE	5	aaTGACAgta ,cggCGTCAcg, CGTCA, cTGACAgc, ctTGACAggc, tcTGACAgca, TGACA, tTGACAgg
MADF	5	acccGGTTAa, caGTAAAtaa, tTAACCgttg
Myb/SANT	5	aaaATATCcg ,aaaTATCCggg, caaATATCtc, caGATATttg, ctACCGGtgc, gTAGGGca, tACCGGtg, taCCGGTg
Myb/SANT;MYB	5	cAACCGgc ,CAACCggc, cacAACCGgc, ccaAACCGcc, ctcCGTTAcgg, gggCGTTAcac, TAACAag, tAACCGtt
Myb/SANT;MYB-related	5	aaaATATCc, aaATATCc, aaATATCcg ,aaATATCt, aaATATCtc, aGATATtt, aGATATttg, caaATATCt, caGATATtt, gTAGGGca
Myb/SANT;trp;MYB	5	cgcGCTGAgca
SBP	4	aaCGAACaa, cgCGGACaa, cgCGTACtc ,CGTAC, gcgcgCGTACtctact, GTACT, tgCGTAAaa, tgGCACGaa, ttGGACGac, ACTCTac
Storekeeper	4	cgGCCGGcgg, ctGCCGGtcc, gcgCCGGCga, tCGGCCgg, tcGGCCGg, tgGGTCGgc
B3;ARF	3	gcgCCGACac, gtGTCGAcgc, gtgTCGACgc, gcCGACAca
CG-1;CAMTA	3	aacACGCGc, ACGCGc, aCGCGC, ACGCGccga, ACGCGcgcg, ACGCGctcg, ACGCGctga, ACGCGg, aCGCGG, ACGCGgcgc, cagACGCGg, cccACGCGc, ccgACGCGc, CCGCGg ,cCGCGG, cgcgCGCGT, gCGCGC, GCGCGt, gCGCGT, gCGCGTact, tcaACGCGc
Alpha-amylase	2	AATAAa, TAACAag
AP2	2	gcgCCGACac, ggtGGCCGcc

C2H2	2	acttGAACT, ctgAGCTGgg
FAR1	2	aacACGCGctga, CACGCga, CACGCgc, cccACGCGctcg, gccACGCGatcg, tcaACGCGccga
GATA	2	acgcGATCGt, cgcGATCGt, cgTGATCga, cgtGATCGa, gCGATCgtg, gCGATCgtga, tcgtGATCGa
Homeodomain;bZIP;HD-ZIP	2	CAATCattc
LEA_5	2	aacACGTA, gatGCATG
MADF;Trihelix	2	cGGTTAac, GGTTAa, gtTAACCg, tTAACC, tTAACCgttg
MYB-related	2	aaaATATCcg, caaATATCtc, caGATATtg
MYB;ARR-B	2	atGGATCtga, tcGGATCcgg
Myb/SANT;MYB;ARR-B	2	AATCT, atGGATCtga, GGATT, tcgGATCCgg
TCR;CPP	2	cacttAAATTtgc, cctTCAAAtg
BES1	1	cgaaCACGTatgca
bZIP;B3	1	cggCGTCAcg
Dehydrin	1	ACGAC, ATCGG, CAGAC, CCAAC, CCCAC, CCGAA, CCGAC, CCGAG, CCGCC, CCGGC, CCGTC, CGGAC, CTCGG, CTGAC, GACGG, GCCGG, GGCGG, GTAGG, GTCCG, GTCGA ,GTCGC, GTCGG, GTCTG, GTGGG, GTTGG, TCGAC, TTCGG
E2F	1	cgCGCGCgta, tcgGCGCGga
E2F/DP	1	gcaCGAAA
E2F/DP;E2F	1	aCGCGCcgac, gtcgGCGCGg
EIN3;EIL	1	ggATGCAtga, ggaTGCATga
HD-ZIP	1	gtatgcAATGC
Homeodomain;bZIP;HD-ZIP;WOX	1	cacCAATCat
LFY	1	acttgacaggCGGTCtacc
LOB;LBD	1	gatCCGGGgc
MADS box;MIKC;M-type	1	aCAAAAgaggc
MYB	1	tCCGTTacgg, tgtTGGTTcg
Myb/SANT;trp;MYB;NF-YC	1	cgCTGAG
NF-YB;NF-YA;NF-YC	1	AATGG, ATAGG, ATCGG, ATTGC, ATTTG, CAAAT, CCAAA, CCAAC, CCAAT ,CCACT, CCATT, CCTAT, CTTGG, GCAAT, GTTGG, TTTGG
TBP	1	aATAAAta
Trihelix	1	ATAAC, CTTAC, GAAAC, GCAAC, GGAAC, GTAAA, GTAGC ,GTATC, GTCAC, GTTAA, GTTAC, GTTGC, GTTTC, TTAAC
VOZ	1	acgaaaggggAACGC, ccctccagccCACGC ,gaggaggctcAACGC, gcatgctccaGACGC, gcgcagccaaCACGC, GCGTActctactcac, GCGTCacgacgatgg, gctcgtcgccGACGC, ggttttcggcCACGC
WRKY	1	TTGACa
ERF	1	gcCGGCT, ggCGGCT
NAC	1	aacGCCGT

Table showing all the binding sites used to predict the TFs which bound to TaWRKY19s promoter. Also shown are the number of unique TFs which are predicted to bind per family.

Supplemental table 5

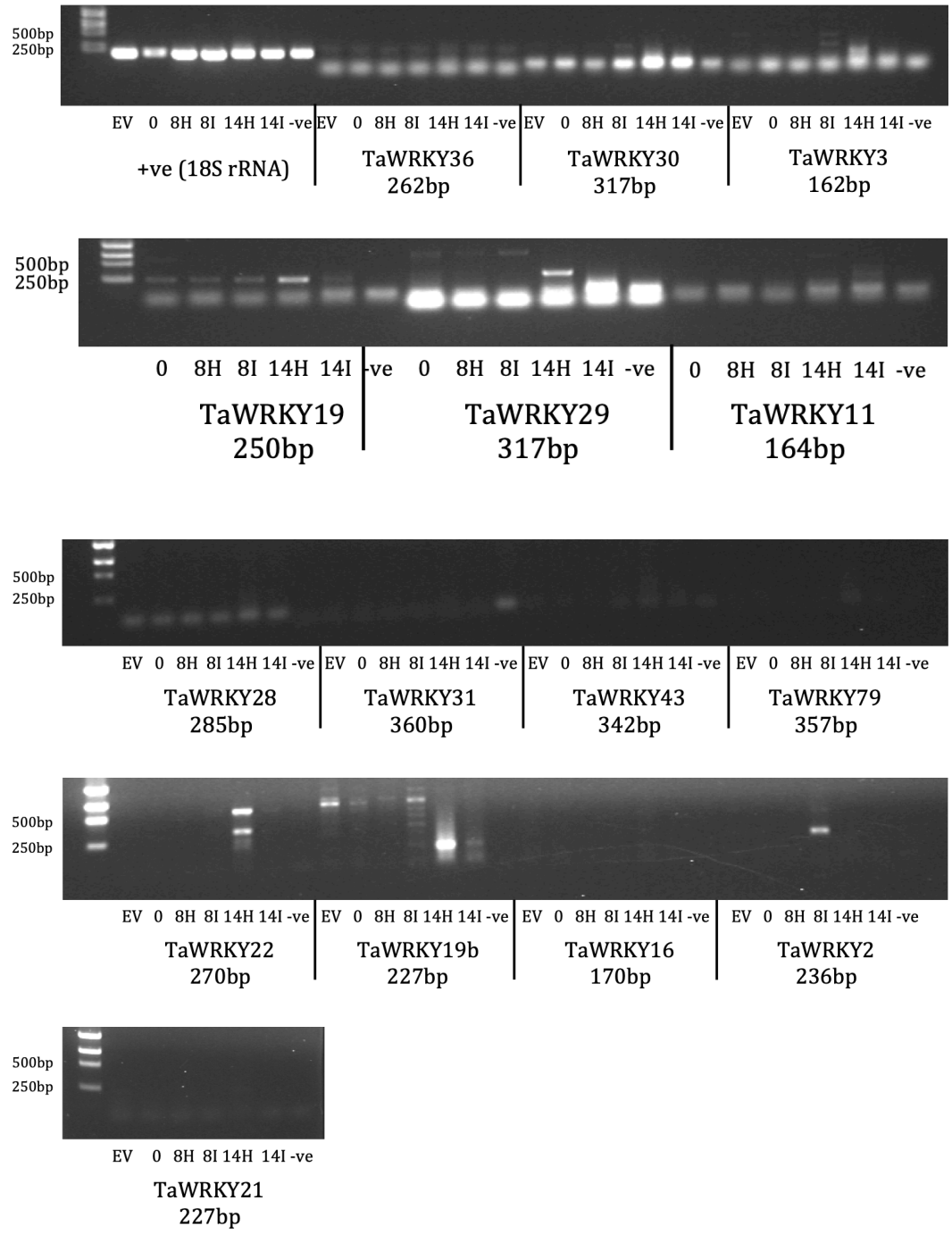
Family	No. of TFs	Hit sequence
WRKY	28	aaaGTCAAct, aaaGTCAActa, aaaGTCAActatg, aaGTCAAc, aaGTCAAct, aaGTCAActa, AGTCA, aGTCAA, aGTCAAct, aGTCAActat, aGTCAAtg, attGTCAAca, attGTCAAcaa, attGTCAAcaata, cccgTTGACg, ccgTTGACgg, ccgTTGACgga, cgaGTCAAtga, cgTTGACg, cgTTGACgga, CGTTGacggac, CGTTGcccctg, gaGTCAAt, gaGTCAAtga, gcccgTTGACgga ,TGACC, tGTCAA, tGTCAAca, tGTCAAcaat, TTGACg, ttGTCAAc, ttGTCAAcaa
AT-Hook	25	aaaAAAAT, aaAAAATg, aaAAATT, aAAATAt, aaacATAAT, aAATATatta, aaataTATTA, aagaATAAA, acatAATAAa, agaATAAA, atAAAATt, ataATAAA, atAATATttg, ataTATTTaa, ATTATatgc, aTTATTgccg, ATTATttcc, aTTATTtcct, atTATTTt, ATTATtttc, attTAATAtttc, attTATTT, caAAAAT, caaaTATATt, caaTATTTat, cAATTAtttt, cataATAAA, cATTTTtt, ccccAAAAA, gagcAAAAA, gcaaAAATT, gttaTATTAata, taagAATAAa, tATATAttta, tattAATAAt, tatTAATAatat, tatTTAATat, tcAAATAtat, tcAATATtta, tcaATATTta, ttAATATttc, ttaATATTtc, ttatTATATg, ttatTTATTt, TTATTttcc, TTTATgcca, TTTATtat ,TTTATtata, tTTATTatat, TTTATttat, tTTATTtatc, TTTATttgt, tTTATTtgtt, TTTTTatctc
Dof	23	aAAAAGc, aAAAAGcgc, aAAAAGt, aAAAAGtgc, AAAGA, AAAGC, AAAGCa, AAAGCg, AAAGG, AAAGT, aacCTTTAttt, AAGGA, AAGGC, AAGGT, ACCTT, ACTTT, aCTTTTt, aCTTTTg, aCTTTTtgg, agaAAAAGt, agaAAAAGtgc, ataaAAAGCgc, ataAAAAGcgc, ataCTTTTt, ataCTTTTg, caAAAAGcat, caAAAGAtgt, ccaaAAAGCat, CCCTT, CCTTT, cGCTTT, gaAAAAG, gaAAAAGt, gaAAAAGtgc, gcCGTTAttg, GCTTT, gtCGTTAttg, gtGCTTTaccc, gtgCTTTAttt, gtGCTTTattt, taAAAAGcgc ,TCCTT, tcgCTTTTc, tcgCTTTTcgg, tCGTTAtt, TCTTT, tGCTTT, tttCTTTTgtt
SBP	20	aaggtCGTACgtacaa, aCGTACaac, AGTAC, caAGTACaa, CGTAC, cGTACGtac, ctGGACGga, ggtCGTACg, ggtCGTACgt, ggtcGTACGt, ggtcGTACGtac, ggtCGTACgtac, ggtcgtaCGTACaacatat, GTACA, GTACG, gtaCGTACa, gtaCGTACaa, gtaCGTACaaca, gtCGTACg, gtCGTACgt, gtcGTACGt, gtCGTACgta, gtcGTACGta, taCGTACa, taCGTACaa, taCGTACaac, tCGTACg, tcGTACGta ,tcGTACGtac, tcgtaCGTACaacata, TGTAC, ttCGAACag, ttCGTGCac, ttCGTTCta, ttGTAGGcc
Myb/SANT	17	aaaATATCat, aaCCCTAaac, accgGAATCa, acCTACCtt, ccgGAATCa, gaGATAAgat, gGATTCcccc, ggCCCTAa, gGGATAag ,ggGATAAga, ggGGATAaga, gggGATAAga, taacCTACCtttc, taagCCGTTa, tggGGATAag, ttggGGATAagaa
AP2;ERF	16	AACTA, AAGAT, ACCTA, aCGCCGgc, acgCCGGCtg, aGCCGTcctc, aGCCGTtatt, ATATA, ATCAA, ATCCA, ATCGA, ATCTA, ATCTC, ATCTG, ATCTT, ATTTA, CACTTccc, CAGAT, caTGTCGacg, cgCCGGCt, cGCCGGct, GAGAT, GTCGGg, GTCTA, TAAAT, TACAT, TAGAA, TAGAC, TAGAG, TAGAT, TAGCT, TAGGT, TAGTT, TATAT, TCGAT, tgcggCACCG, TGGAT ,tGTCGGga, TTCTA, TTGAT, ttGCCGCc, ttgCCGCCa, ttGCCGCcag, ttGTCGGgac
bZIP	11	AAGAAt, ACACCagt, ACAGCt, ACAGGtgg, ACCGCt, acgCATGT, ACGTA, ACGTG, ACGTT, actACGTGcttta, ACTGCt, agaAGTGT, agaCAGCTcg, aGACAGctcg, AGACG, agAGCTGgca, aGCCGT, aGCGGT, AGTCA, ATGACatctt, CATCA, CCACGaga, CCTCA, CGACG, CGCCA, CGGCA, CGTCC, CGTCT, CGTGA, CGTTA, ctcgCTGTCc, CTTCA, gcaTGTGT, gGACAGcgta, gGACAGgtgg, GGACG, gtTGACGgac, TACGT, TCACG, TGAAG, TGACA, TGACC, TGACG ,TGAGG, TGATG, TGCCG, TGGCG, TGTCA, TGTCG, TTACG
GATA;tify	10	AATCA, AATCC, AATCG, aatcGATCTg, AATCT, AGATA, AGATC, AGATG, AGATT, ATATC, atcGATCTga, cAGATCaaaa, caGATCTt, caGATCTtg, cAGATCttgg, CATCA, CATCC, CATCT, CGATA, CGATC, CGATG, CGATT, CTATC, GATAA, GATAC, GATAG, GATAT, GATCA, GATCG, GATCT, gcAGATCaaa, gcAGATCtt, gcaGATCTtg, gcAGATCttg, gcaGATCTtgg, GGATA, GGATC, GGATG, GGATT, GTATC, TATCA, TATCG, TATCT, tcGATCTga, TGATA, TGATC, TGATG ,TGATT, tgcaGATCTt, tgcAGATCttg, tgGATCTga, TTATC, ttgGATCTga, tttgGATCTg
NAC;NAM	10	aaGTCAAct ,CATGTg, gaGTCAAtg, ggtgGCGTGa, gtgGCGTGa, gtgGCGTGaa, tgGCGTGaat, tTTACTtca
Others	8	ACCGAaa, CCGTTa, CCGTTg, CGGTTa, CGGTTg, CTGTTa, CTGTTa, GACATc, gACATC, GACGAc, gACGAC, GACGCc, gACGCC, GACGCc, gACGCC, GAGGTc, gAGGTC, GCCGTc, gCCGTC, GTCGTc, gTCGTC, TACGTa, tACGTA, TACGTg, tACGTG, TACGTt, tACGTT, TAGGTa, tAGGTA, tatagGTGGA, TCCACcggga, TCCGTa, tCCGTA, tcTCGGT, TGCGTa ,tGCGTA, tgctagACGGA, tgTCGGG, tgTCGTT, tgTGGGT, tgTTGGT, ttTTACA
Homeodomain ;HD-ZIP	8	caTTATTgc, atgTGATTga, cagTGATTgg, atgTGATTga, cagTGATTgg, gTGATTga, gTGATTgg, gaAATCAtag, aATTATtt, gTGATTgatt, gTGATTggtt, tggATTTA, tatATTTA
AP2;B3;RAV	7	aCAACAta, ctttTGTTGgtt, gaTGTTGc, gggaCAGGTggc, gtaCAACAta, tattTGTTGgaa, ttTGTTGt
bHLH	7	aCAAGTtga, actACGTGct, ATACAagt, ATACTttt, ATAGGtgg, ATGCGtat, atgCGTAT, ATGCGtgc, CAATTg, cAATTG, CACGAg, cACGAG, CACTTg, cACTTG, CAGGTg, cAGGTG, CATGTg, cATGTG, ctaCGTGC, CTACGtgc, gaaCATGC, GCACGccc, GCACTtcc, GCACTtgc, gcaCTTGC, GCATGtgt, gccCCTGC, gcgCTTGC ,GTACGtac, gtaCGTAC, TACGTg, tACGTG, tcaCGCAT, tcgACTTGa, tgcACTTGc
TBP	7	aaTATAT, ATATAtt, atattgtATATAtttaatatt, gaTTTATa, ggaTTTATagg, gttaTAAAAa ,gttATAAAaaa, taTTTATc, taTTTATg, taTTTATt, tggctggaTTTATag, ttATAAAaaaatgca,
Myb/SANT;MYB	6	aACCTAcc, agcCGTTAttg, ctaACCTAcct, TAACAat, taacCTACCt
SRS	6	gaAGAGT, ttAGAGT
TCP	6	ACCCG, ACCCGc, ACCCGg, AGCCC, AGGCC, CGACC, CGCCC, CGGCC, CGTCC, ctaGGCCC, GAACC, GACCC, gacGGACC, GAGCC, gagGGACA, GCACC, GCCCC, GCCCG, GCGCC, gcgGCACC, GCTCC, GGAAC, GGACA, GGACC, GGACCcgc, GGACG, GGACT, GGAGC, GGATC, GGCAC, GGCCA, GGCCC, GGCGC, GGCTC, GGGAC, GGGCG, GGGCT, GGGGC, GGTCC, GGTCCttc, GGTCG, GGTCGtac, GGTCT, GGTGC, GGTTC, gtaGGCCC, GTCCC, GTGCC, gtgGGAAC, gtgGGACA ,gttGAACC, TGACC, TGCCC, TGGCC, tggCCCACga, tgGTGGGaca, TGTCC
B3	5	AATGC, ACATG, atatGCATGgt, atGCATGg, CAAGC, CACGC, CAGGC, caGTGTAcag, CATCC, CATGA, CATGC, CATGG, CATGT, CATTC, CCATG, CCTGC, CGTGC, CTTGC, gagtTGTAGg, GATGC, GCAAG, GCACG, GCAGG, GCATA, GCATC, GCATG, GCGTG ,GCTTG, GGATG, GTATG, TATGC, TCATG, tCTACAccga, ttcTACACcg

C2H2	5	aAGTGTc, cACACTg, cAGTGTa, gagAGCTGgc, gAGTGAg, gtCAGCTtca
Homeodomain ; bZIP ;HD-ZIP	5	cagTGATTgg, gctcaATTATtttc, cgaccATTATtgcc, ccaTTATTt, caaTTATTt, ccaTTATTg, ataTTAATaa, atATTAAtaa, CAATGatgg, cagtGATTG, CAATTattt, caaaAATTG, CAATGcttg, ccatTATTG
Homeodomain ;HD-ZIP ;bZIP	5	caATTATt, ggctcaaTTATTt, acgaccaTTATTg, ctcaaTTATTtt, gaccaTTATTgc, gctcaaTTATTttc, cgaccaTTATTgcc, caaTTATTt, ccaTTATTg
Myb/SANT;MYB-related	5	aaaATATCa, aaATATCa, aaATATCat, aaCCCTAa, ggCCCTAa, tGATATtgt
B3;ARF	4	atGTCGAcgc, ttGTCGGgac, gAGACA, gtTGTCGgg
MYB-related	4	aaaATATCat, aaaATATCat ,aAGATAtgca, ccCTAAA, ggcaTATCTc, tccgTATCTt, tcCTAAA, TTTAGag, TTTAGga
Myb/SANT;MYB;ARR-B	4	AATCC ,AATCT, AGATT, gcAGATCttg, gcaGATCTtg, GGATT, tccGTATCtt, ttGGATCtga
Alpha-amylase	3	AATAAa, AATTAga, acTGTTA, TAACAat, tcTAATT, tTTATT
GATA	3	cgTGATCag, gtGATCAgg, tCGATCtga, tCGATCtgaa
Homeodomain ;TALE	3	aaTGACAtct, aaTGACAtct, aTGACAtc, AGTCA, TGTCA, TGACA, AGTCA, TGACG, AGTCA, TGACA, TGTCA, TGACC
MYB	3	aACCTAcctt, gCCGTTattg, taACCTAcct, tgtTGGTTat
MYB;ARR-B	3	ccgTATCTtc, gcAGATCttg, gcaGATCTtg, taAGATAtgc, ttGGATCtga
AP2;B3	2	tttACGTTga
AP2;RAV;B3	2	CAACA, cAGGTG, TGTTG
bZIP;Homeodomain;HD-ZIP	2	caATTATttt, ccATTATtgc, ctaATTATga
Homeodomain	2	agTTCATg, taaTAATAtttgg, tgattTATTAtat, aaataTATTAtac, acaTAATAaaatt
Homeodomain ;bZIP ;HD-ZIP ;WOX	2	gTGATTga, gtGATTGatt, gtGATTGgtt
HSF	2	GGAACtttcc, tGAAGCttcg, tgaaGCTTCg
Myb/SANT;ARR-B	2	ccgtATCTTc ,gAAGATgcga, gaagTATCTgata, gataAGATAtgca, gcaGATCT, ggcaTATCTccgg, tccgTATCTtcca, ttagAGATAagat
AP2	1	TCGTAcgtac
AP2;RAV	1	aCAACAta, gaTGTTGc
BES1	1	CGTGCg
CG-1;CAMTA	1	CCGCGc
Dehydrin	1	ACGAC, ATCGG, CAGAC, CCAAC, CCCAC, CCGAA, CCGAT, CCGCC, CCGGC, CCGTC, CCTAC, CGGAC, CTCGG, GACGG, GCCGG, GTAGG, GTCAG, GTCGA ,GTCGG, GTCGT, GTCTG, GTGGG, GTTGG, TCGAC, TTCGG
E2F/DP	1	TTTCCcac
EIN3;EIL	1	ataTGCATgg
ERF	1	AGCCGtc, gcCGGCT
FAR1	1	CACGCcc
GATA;Dof	1	AGATCaa
GRAS	1	cGTACGtac, gtaCGTACa
HB-PHD	1	CTAATcttttg, CTAATtatgaa
Homeodomain ;WOX	1	gTGATTga, gTGATTgg
Homeodomain ;ZF-HD	1	tcTAATCcca, gcTAATCttt
LEA_5	1	cacGCATG, tatGCATG
LFY	1	gtaggccctcCGGTCcttc,
MADF	1	cTAACCcggc
Myb/SANT;G2-like	1	ccgGAATCaa
NF-YB;NF-YA;NF-YC	1	AATGG, ACAAT, AGTGG, ATAGG, ATCGG, ATGGG, ATTAG, ATTCG, ATTGA, ATTGC, ATTGG, ATTGT, ATTTG, CAAAT, CCAAA, CCAAC, CCAAT, CCACT, CCATT, CCGAT ,CGAAT, CTAAT, CTTGG, GCAAT, GTTGG, TCAAT, TTTGG
PsaH	1	tccctttGTTTA
Sox;YABBY	1	ctAATTAtga
TCR;CPP	1	taataAAATTtac
Trihelix	1	ATAAC, CTAAC, GAAAC, GATAC, GCAAC, GGAAC, GTAGC ,GTATC, GTTAC, GTTAT, GTTGC, GTTTC, TTTAC, tTTACC

trp	1	acCTACCtt
VOZ	1	gagccatgtcGACGC, gcgcttgcatCACGC, GCGTAtgctagacgg
ZF-HD	1	ATAAT, ATTAA, ATTAT, CTAAT, TTAAT

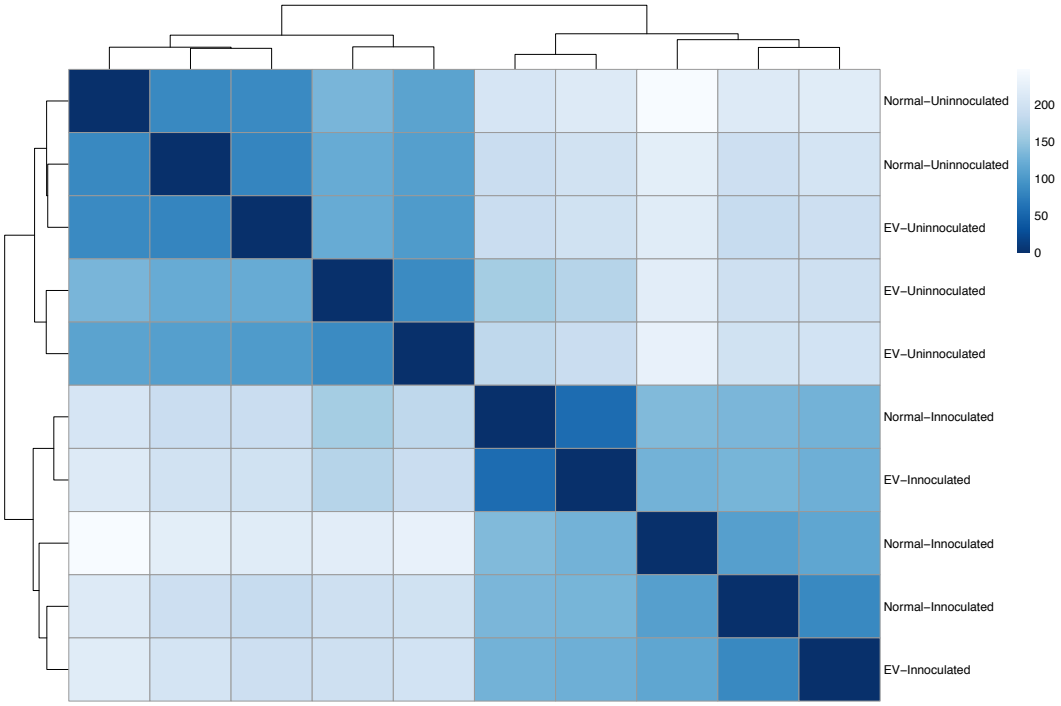
Table showing all the binding sites used to predict the TFs which bound to TaWRKY9s promoter. Also shown are the number of unique TFs which are predicted to bind per family.

Supplemental figure 1



Gel pictures of semi quantative PCR showing the expression levels of 15 WRKY genes in healthy and infected wheat (0d, 8d and 14d). Negative controls are included in which no cDNA was added to the PCR mix. The expected size of the DNA fragment is also shown.

Supplemental figure 2



Heatmap representing the relationship of samples based on genes expressed obtained from RNA sequencing. Two samples were removed due to them being outliers (BSMV:TaWRKY19, infected and BSMV:00 uninfected). Samples are labelled on the right hand side. Darker blue shades represent higher levels of the same genes being expressed to a similar level between samples. Samples labelled 'normal' = BSMV:TaWRKY19B, 'EV' = BSMV:00, 'uninnoculated' = uninfected and 'inoculated' = infected.

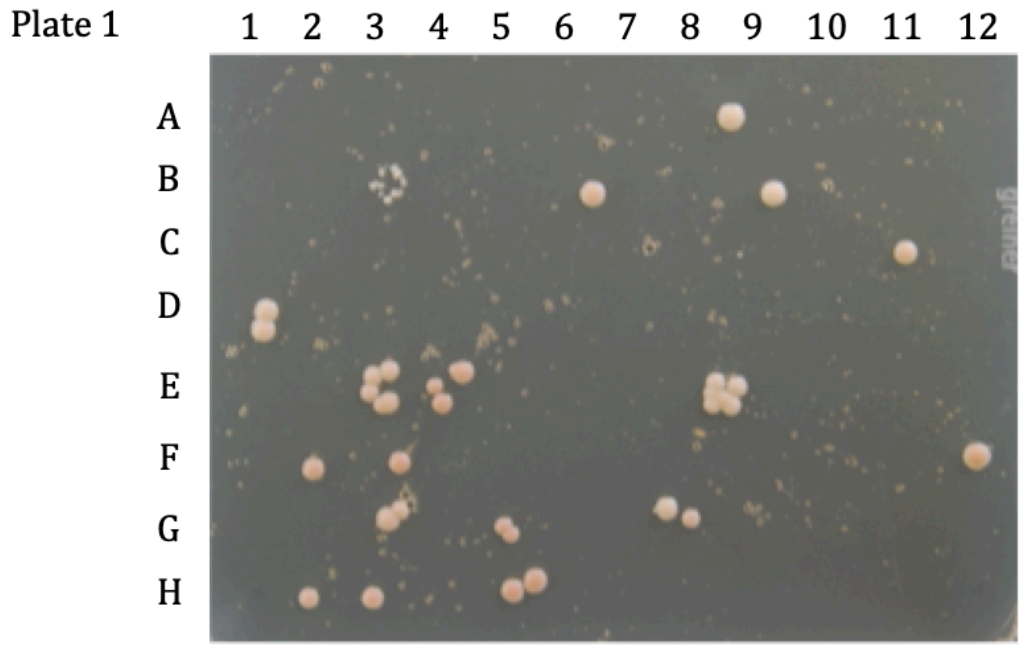
Supplemental figure 3

CAAATGAGGAGCGGTTTTCGGCCACGCGATCGTGATCGAACGACCGAGAG
CATGGAGGGGAGTTTGCTGGGTTTTTGGGCCACTTTGGAGGGGTGTTGGG
CTGCAACACAAAAGAGGCCTTTGCAGTTACCCGGTTAACCGTTGGAGTAT
CAAACGACCTTCAAATGGCACGAAACTTGACAGGCGGTCTACCGGTGCTA
TAACAAGGCCACTTGGCAAGCCTCGGGCCATTCCGAAAAAGTTTAACACC
TGCACACAACGAGAGACGAAAGGGGAACGCCGTAGGGCATAGGAGAGCCG
GATTGCAAAACGAACAACGGGGAAAAGGCTCGGATGCATGAGACGAACAC
GTATGCAATGCAATGCACATGATGACATGATAAAATGCAACACGCAAGCA
AATGACATGGCAATGACAGTAAATAACTAGCAGACACCTGGCGCATCGGA
TCCGGGGCGTTACACCCTTCCCCCCGGCACCGAACACAGAAGCTGGGGTT
CCGGCACTCCGCCGAGATGTGGCCATCATCCTTGCCACAGTTGTAGCAGC
AGCCAAAAAGCTCCGCCGGCATCCTGGACTCCGGCGGCGAACGGGGCGCA
GCCAACACGCGCTGAGCATGGCGGCGCCTCTGTGGGAAGGCAGGGCGAGC
AGGCGGCCCTCTCGGCCGGCGGCTCCTATCATCCAAACCGCCTCCACCTG
GGGTGAATGAGAAGGACCTGCAGCCCCGCCCTGGACCAGCGGACCGAAGG
CCGTGCCCACAACCGGCGTCACGACGATGGATCTGAGCTGGGGGCGGGGG
GGACGGCAGGACGGGCAGAGCATGCTCCAGACGCGGCGCCGGCGAGGAAG
TAGGGGAGGAAAGCTCCGACGAGGATGAGCCGGACAGAGAAACCCGCGAA

GCCATGGCGAGGCGGCGGGGGAAGGGGTGGGGTGGGGTGGCTGCCGGTCC
CGGAGCGGAGCACGGCGCAAGGCTGGTGGCCGCCGGCTGTGTGGGAGAAA
GCACTGCGTAAAATACTTGAACTATAAGAGCAACTCCAATGGAGCGATCC
ATTTCGTCTGCCGCTGTTCGTTTGGGTCGGCGCGGACAAAAGAGGAGGCT
CAACGCGCCGACACAAACCAAATCATGTCCGCTTCGTGTCCGTGTCGACG
CATTTGCACTTAAATTTGCGCCCCAAAGTTACACACAGATATTTGGGTTC
GGTCTGTTGGTTCGGCTTACCATGTTTAGAAGGGGTGGACAAATATCTCC
GTTACGGTTTCGGAAAATATCCGGGTTTTTCCTTCCGCGGGGCTTCGCCT
CCGCTTGGACGACGTGGCACGAGCCACCAATCATTCAGCGCCCTCATTGC
GATTTTTGTTGCCTCGCCCACTCGCACTCCCGTCCGGGGGAATAAATACC
GAGCGTCCCACTTGGCAACCAATCTGACAGCAGAAAGCATCCCTCCCTCC
CTCCAGCCCACGCGCTCGTCGCCGACGCGCGCGTACTCTACTCACCC

DNA sequence for TaWRKY19 promoter region, identified through database searches (KWS and the Earlham Institute[151])

Supplemental figure 4



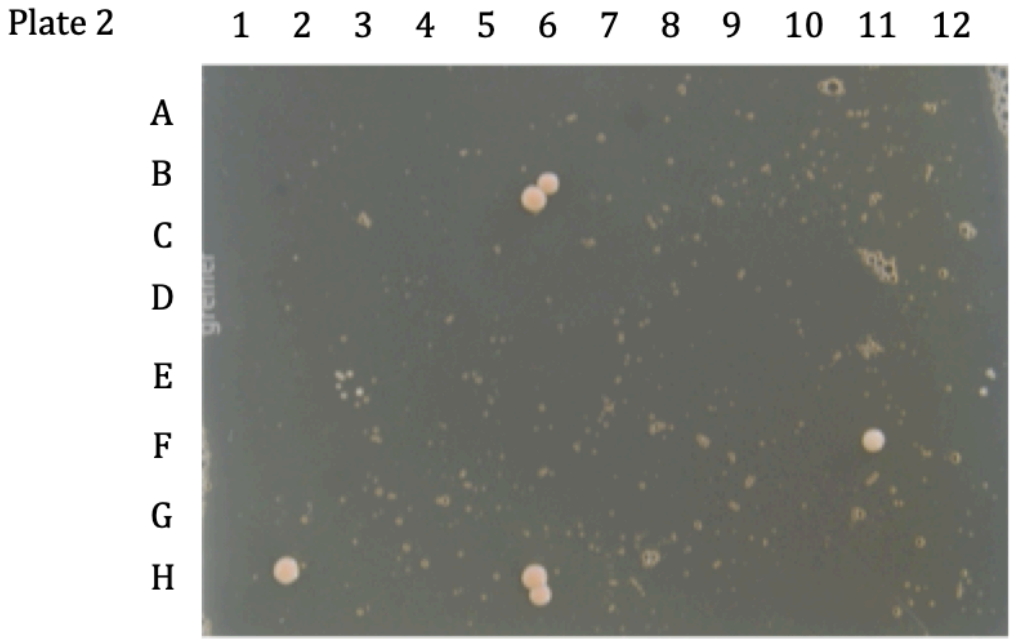


Plate 3

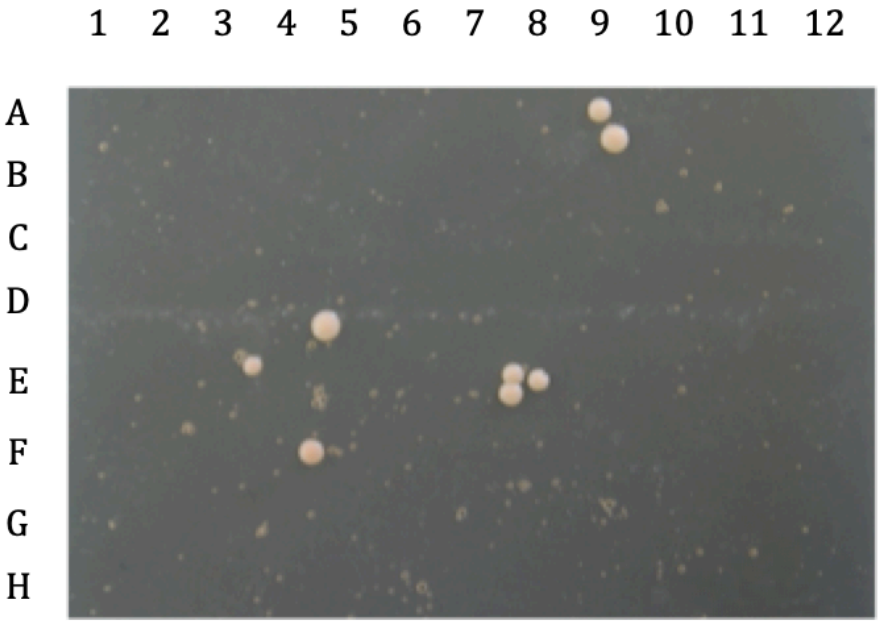


Plate 4

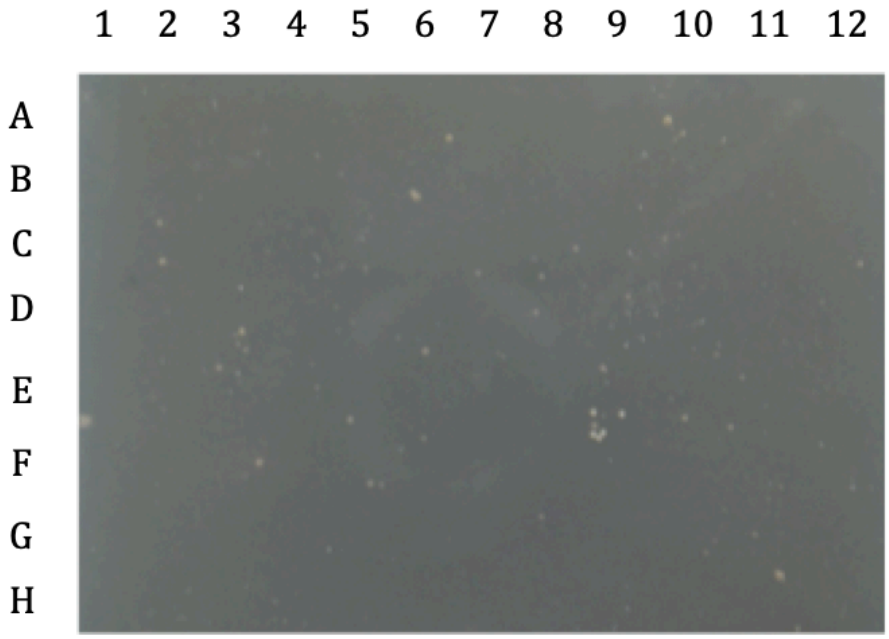


Plate 5

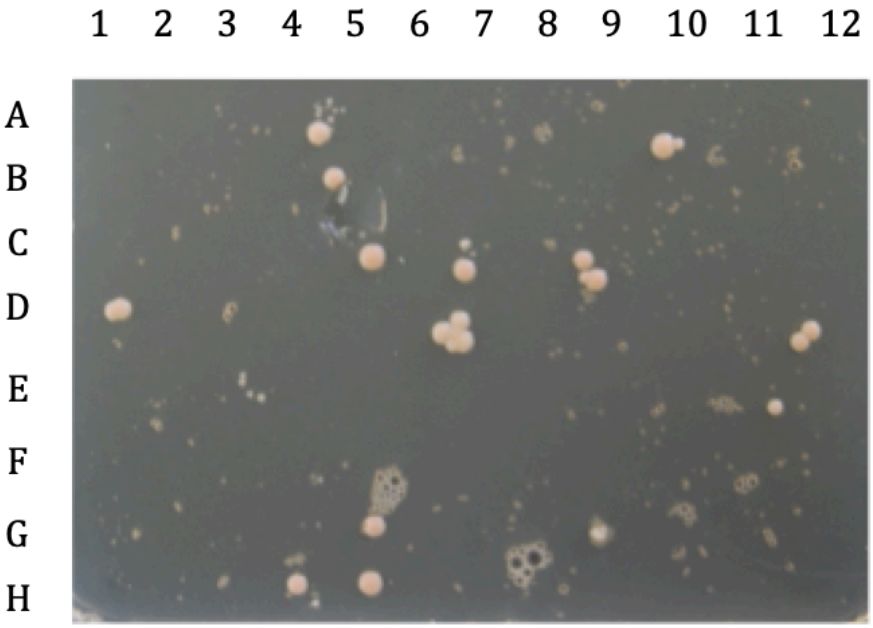


Plate 6

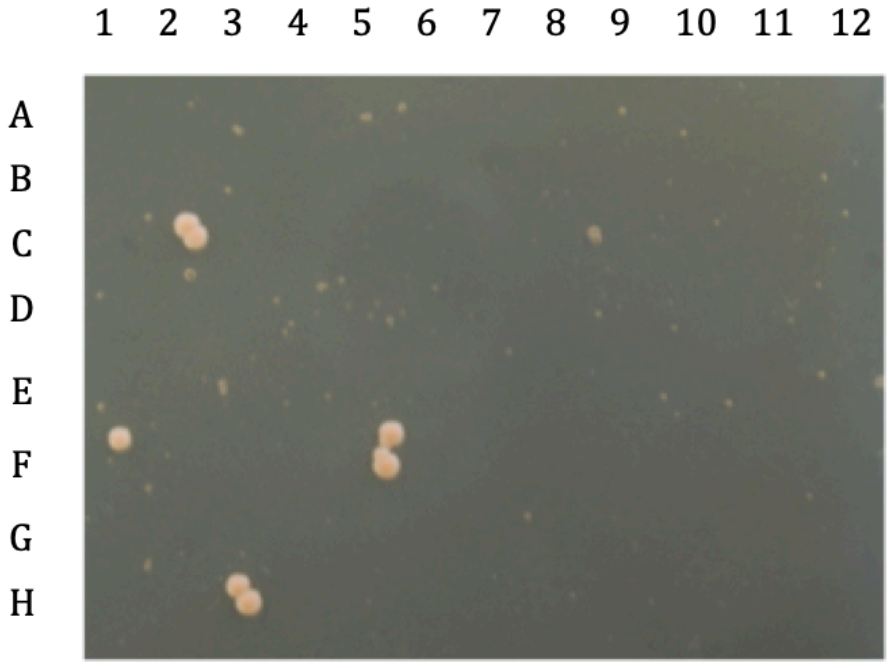


Plate 7

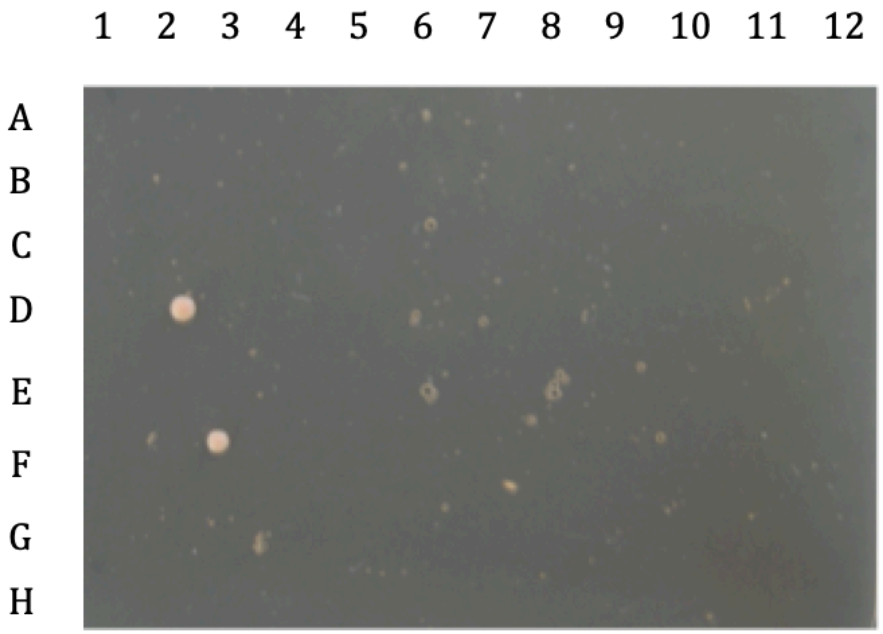


Plate 8

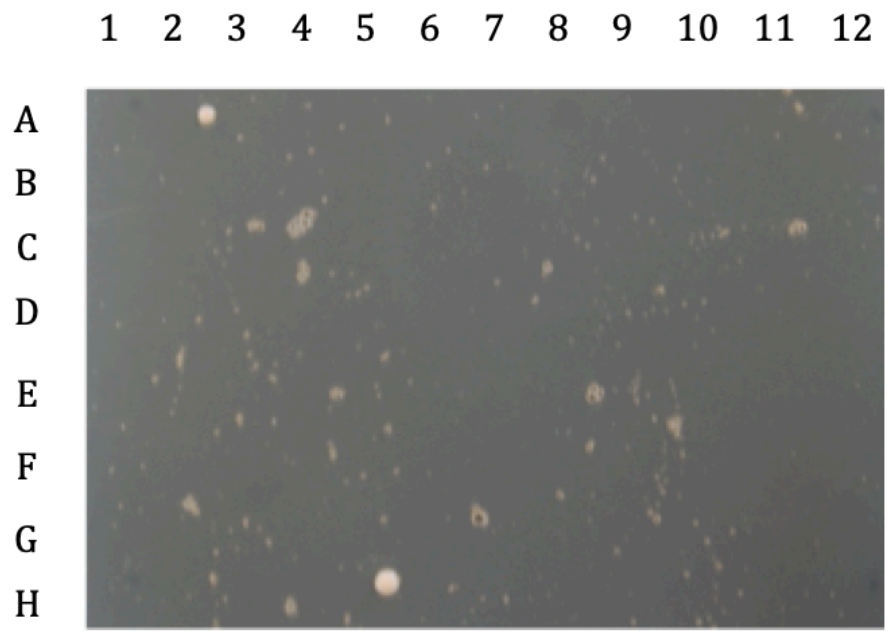


Plate 9

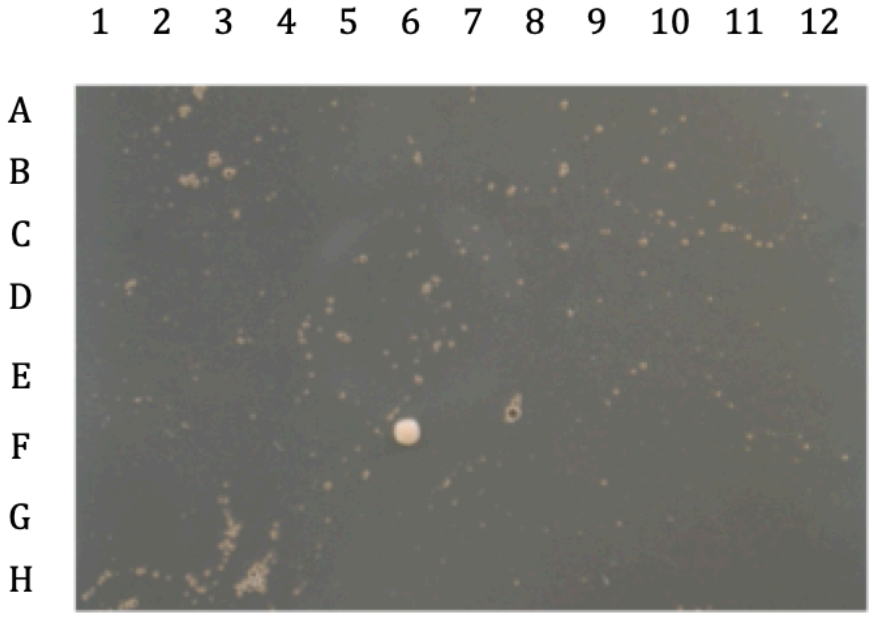


Plate 10

Plate 11

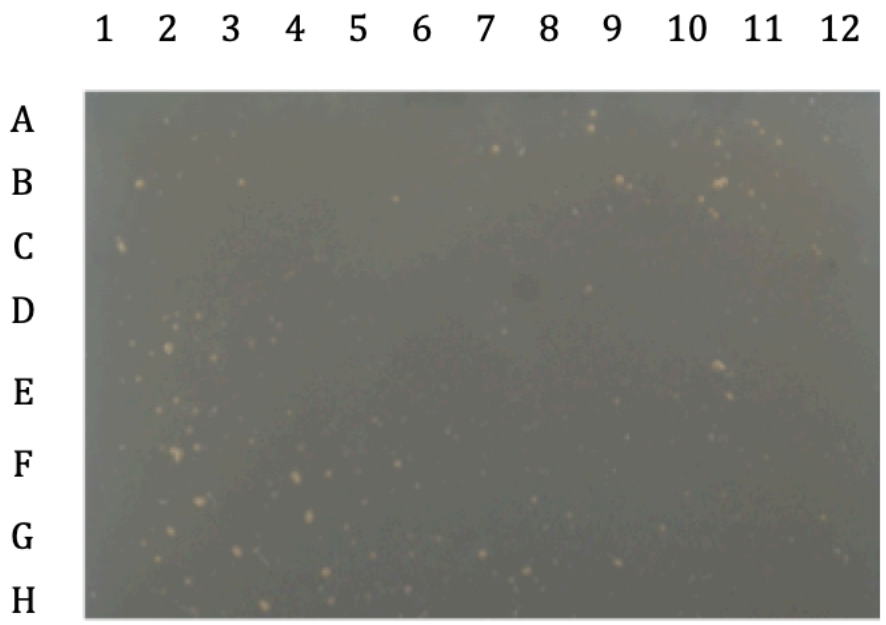


Plate 12

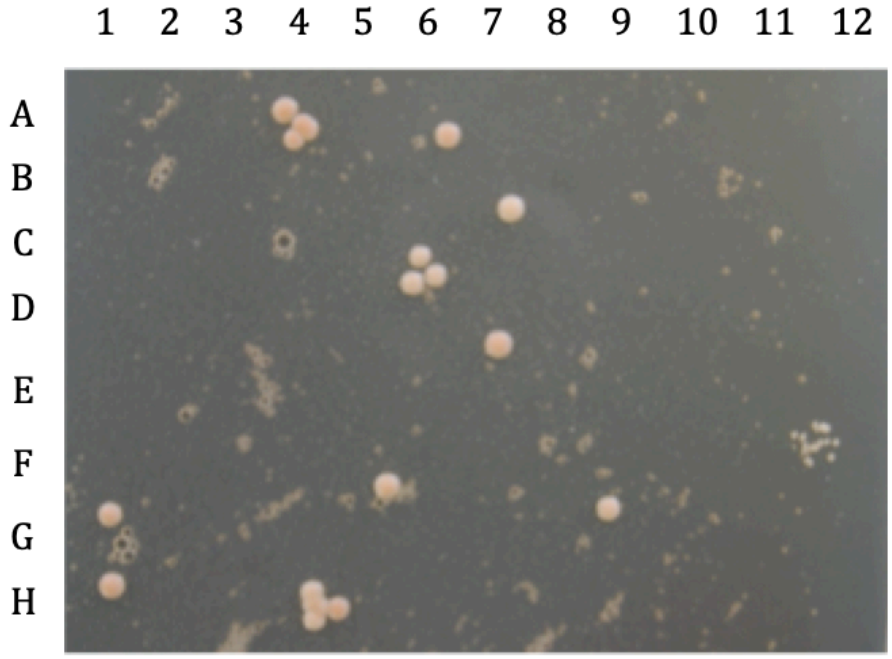


Plate 13

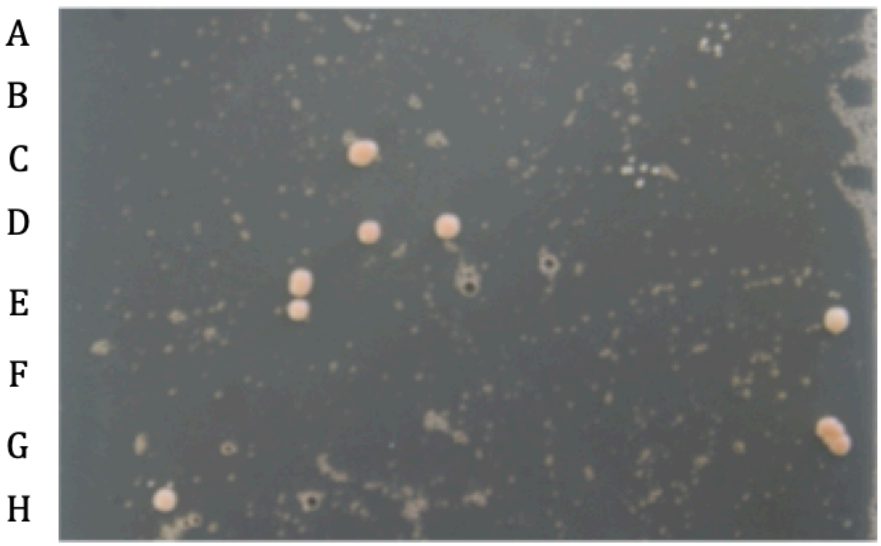
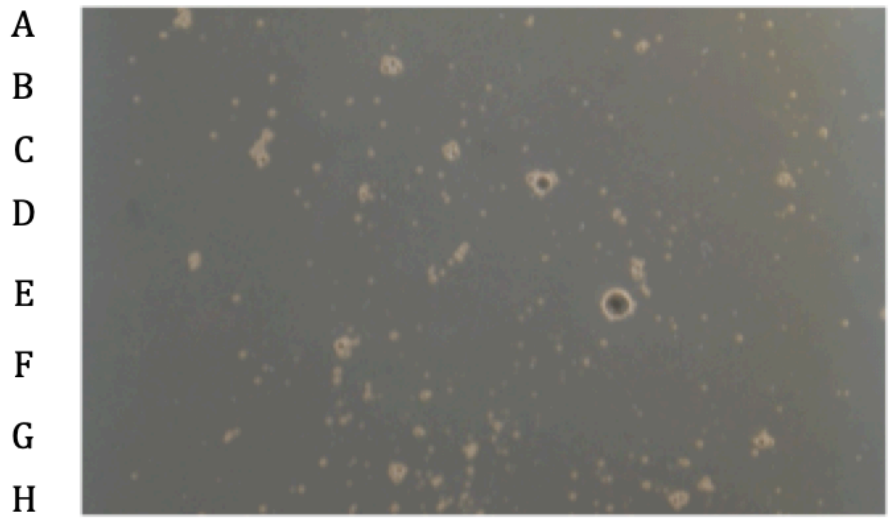
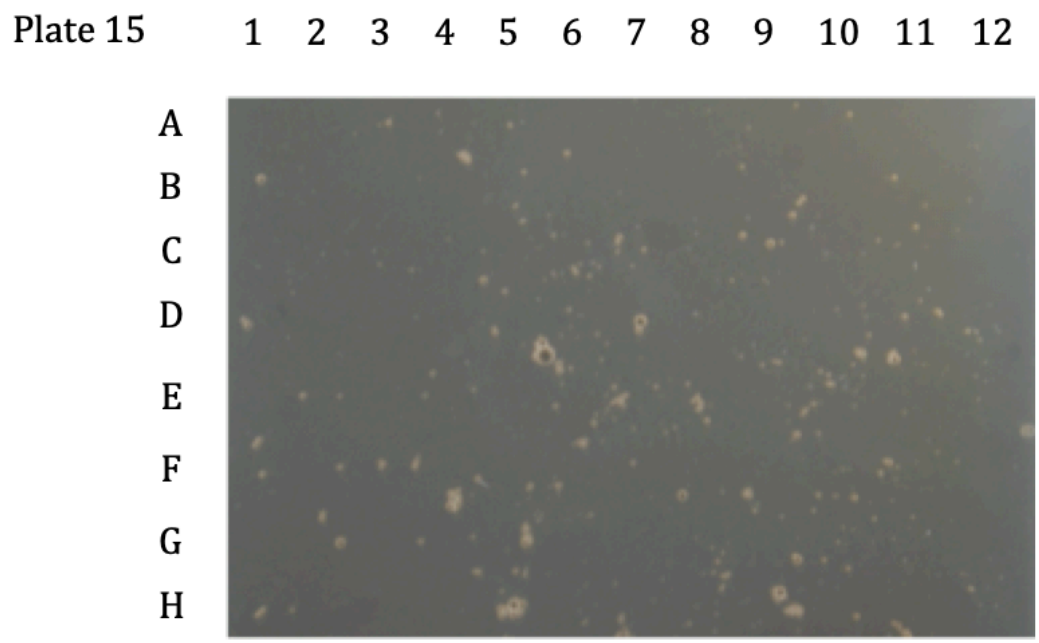


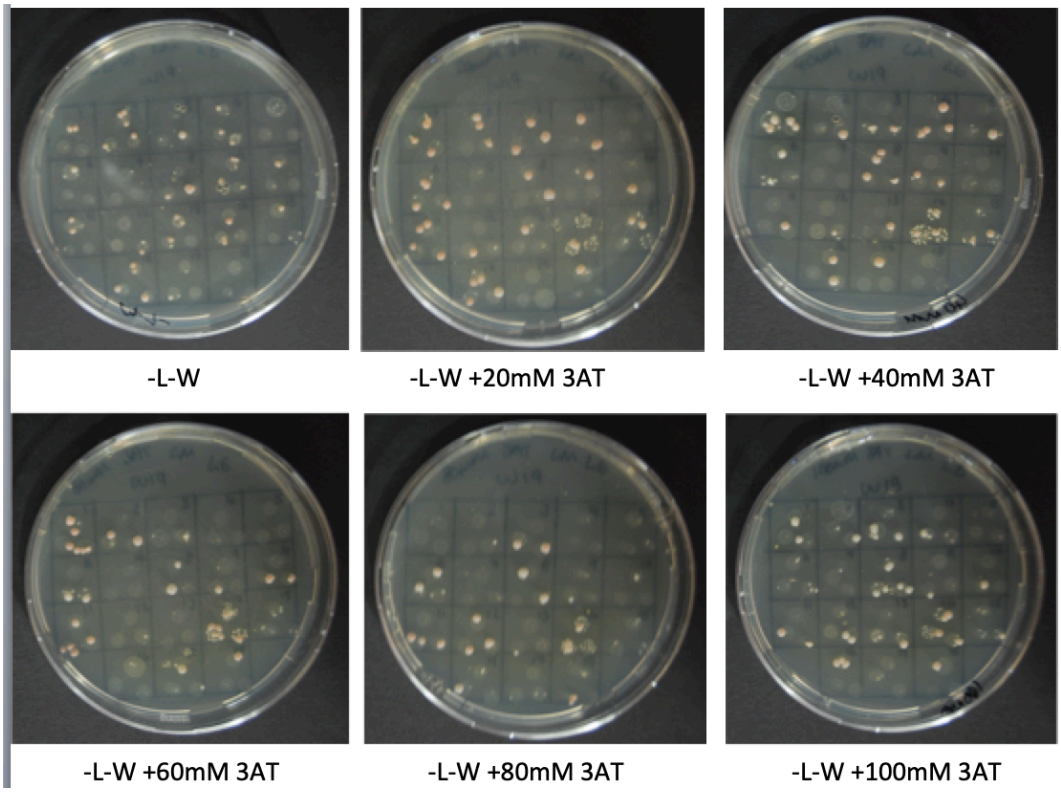
Plate 14

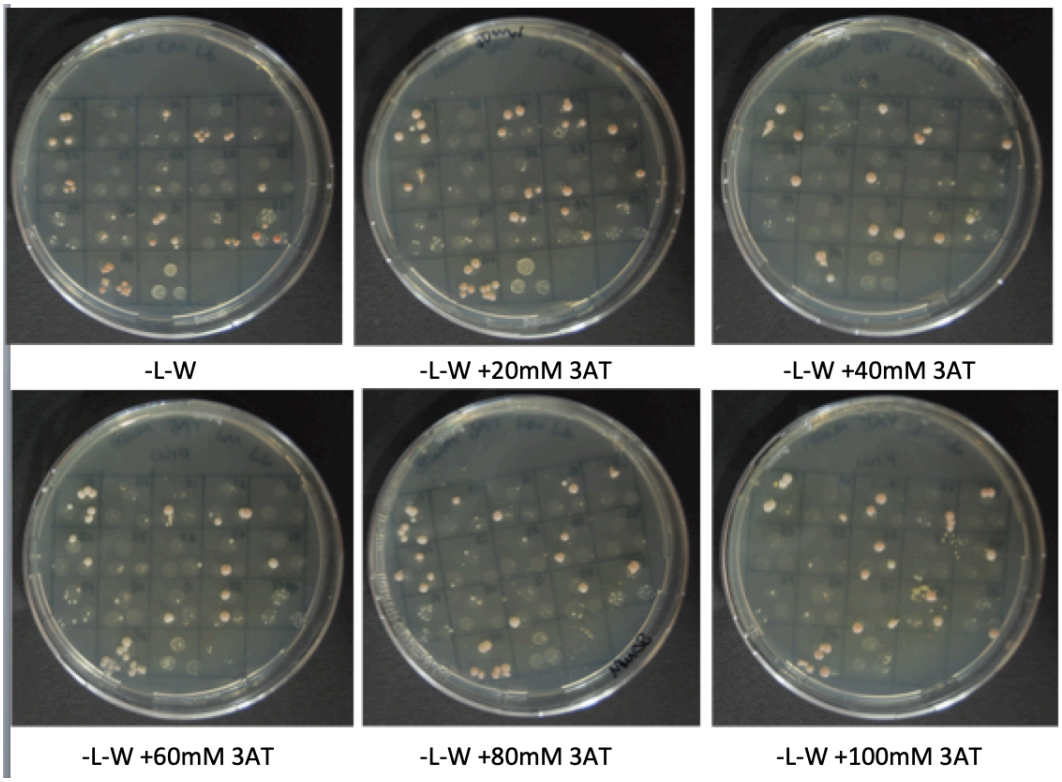




Y1H with TaWRKY9 promoter and a library of 1,200 Arabidopsis thaliana TFs. After mating the yeast was grown in liquid culture (with mating selection) before being stamped onto plates containing selection media (20mM 3AT in –L-W-H minimal SD media). Plates were grown for 4 days at 28^{O}C before photos were taken.

Supplemental figure 5





Pictures of Y1H plates for TaWRKY19 promoter and 34 TFs selected based on preliminary screening. After mating the yeast was grown in liquid culture (with mating selection) before being pipetted (in 5µl) onto plates containing increasing selection pressures (0-100mM 3AT in –L-W-H minimal SD media). Plates were grown for 4 days at 28^{0}C before photos were taken.

Supplemental figure 6

AGTTTAGTTTAGGGTTTTCTAGTCCTTGCAGGGGCAACGCTAGAACGAATGGTG
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CGCAGCCCCAGGGCCTAGTATTTAGGAGGGCGTGCACGAAGCTTCATGGCTATTG
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GTTCTGGCGGCAATAATGGTCGTTTTGTGCTCCTAATACCTCAACGTAAATTTTA
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CAATAACGGCTTAAAGAAAACCCATTTATTCTTATCCCCAAAGTCCACTATCTCG
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AAGCCCCCGCAGCTACTATGCCTCACACCCACAGGTCCTCTGTAACAGTAAACAA
CCAATCACTGTACACTGGCATAAATATTGAAAACAAAATCTAGTAAAATCAAAT
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TTTTTTTATAACAGTGTGAAACTAGACTGGTGTTCAGATCCAAACAGTGGGATT
AGACCGCTCATGTAAAAAATGTGAAGCTGACACTTCTCAAAGAAGGACCGGAGG
GCCTACAACTCTAATTCTGGGGGAATCCAGGGGGCTGCGCCGAAAAGCGAACACC
ATCATTGACTCGCATCTTCTCCCCTCACCCCGTTTCCACCTATAAATCCAGCCATG
GCCTCCGTCCAGACCTCCAACTCCCAACCAAACCGAGAGCGCAACCGGGACAGCG
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DNA sequence for TaWRKY9 promoter region, identified through database searches (KWS and the Earlham Institute[151])

Supplemental figure 7

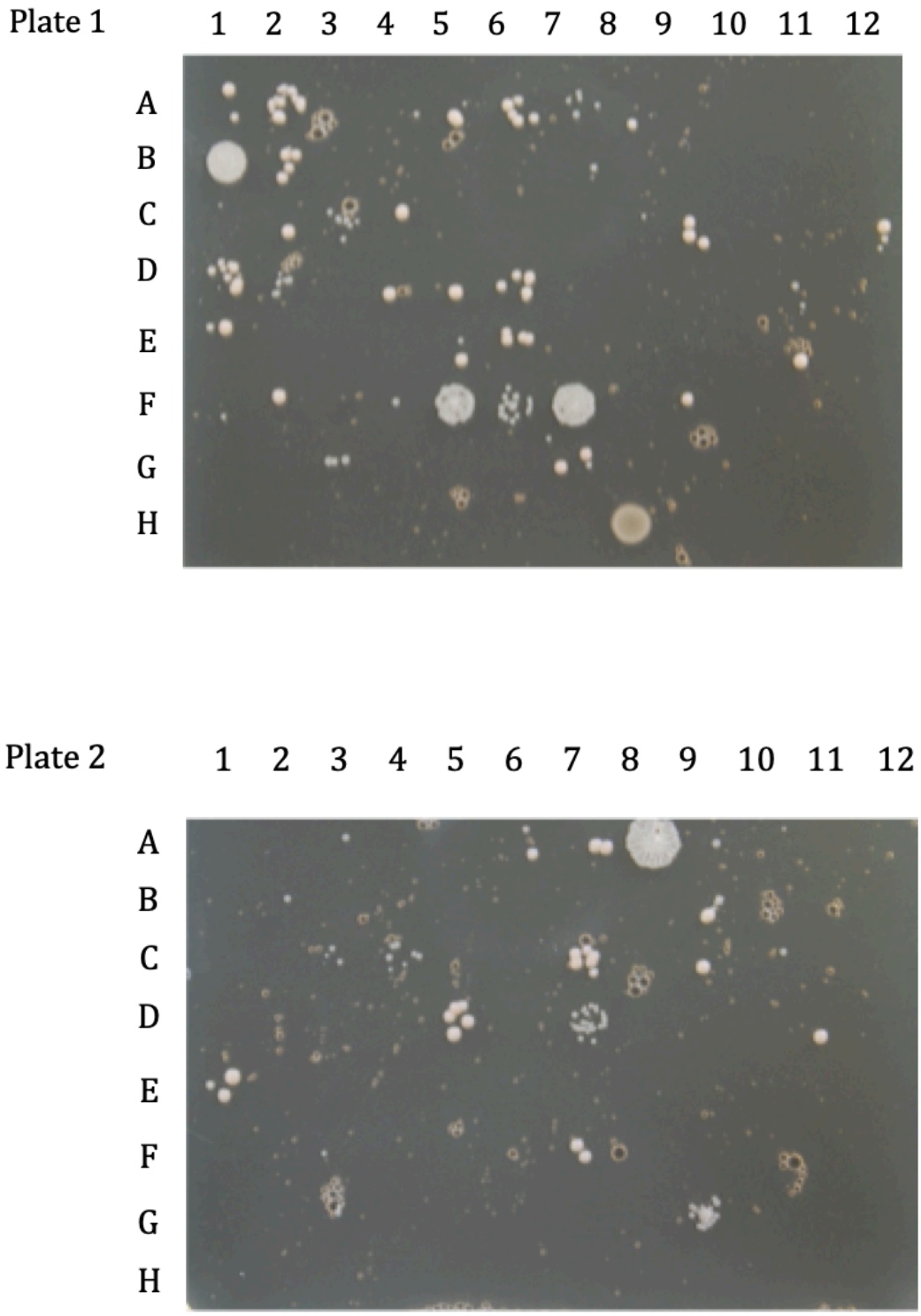


Plate 3

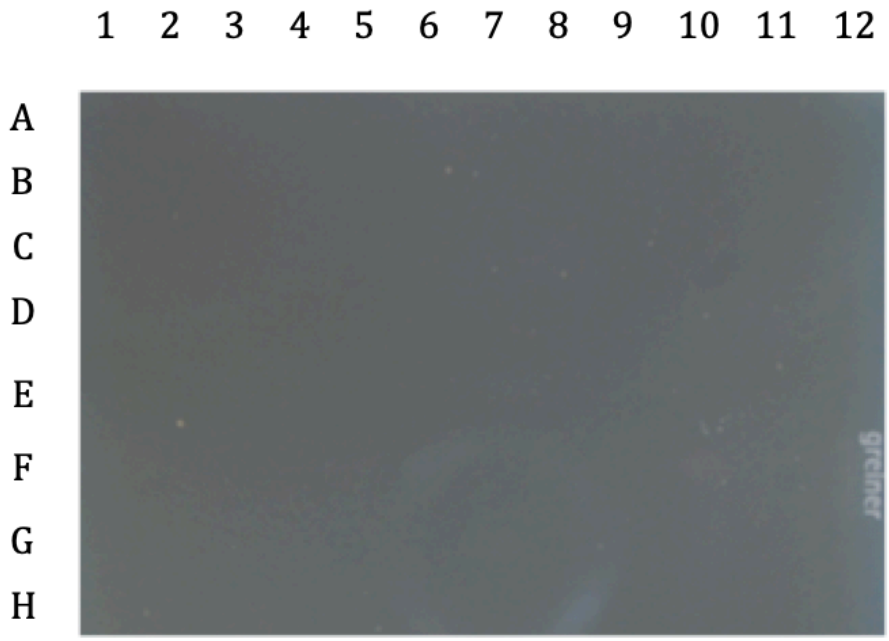


Plate 4

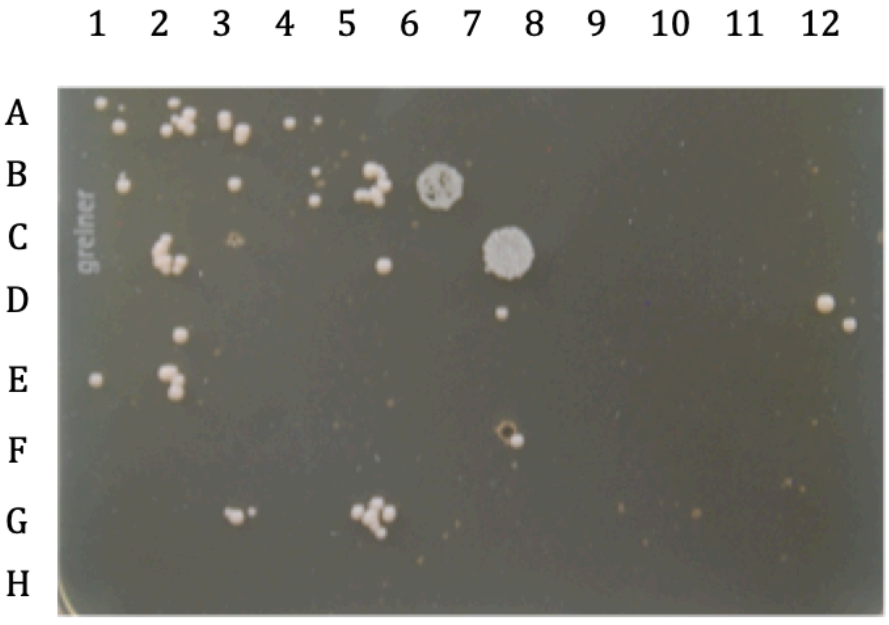


Plate 5

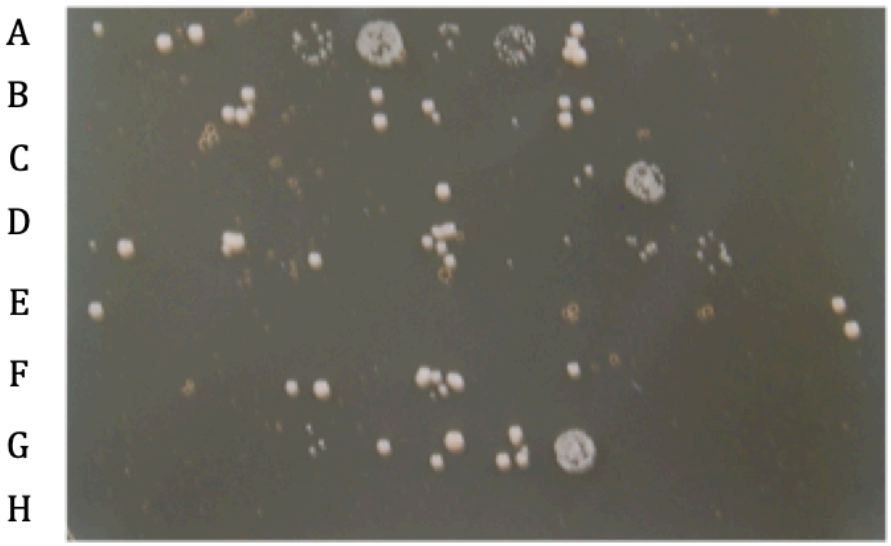


Plate 6

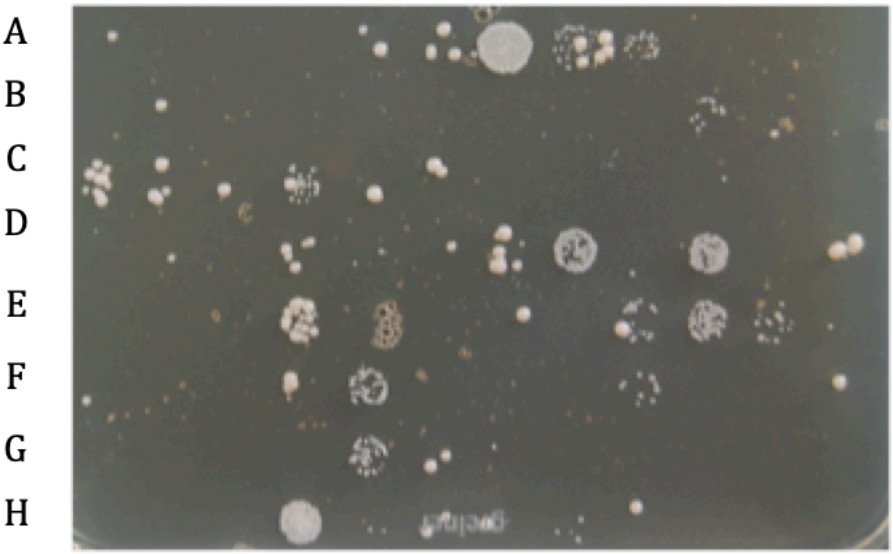


Plate 7

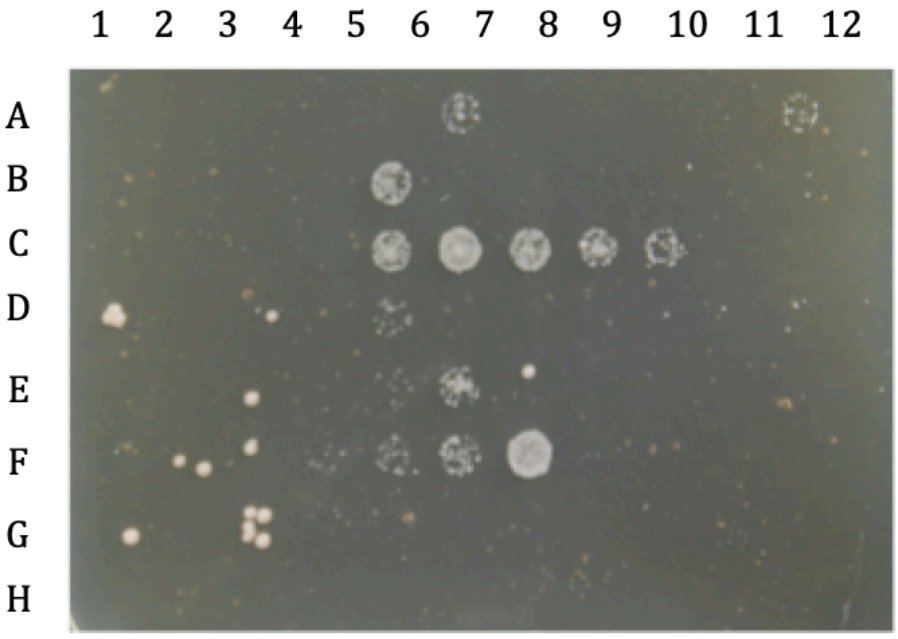


Plate 8

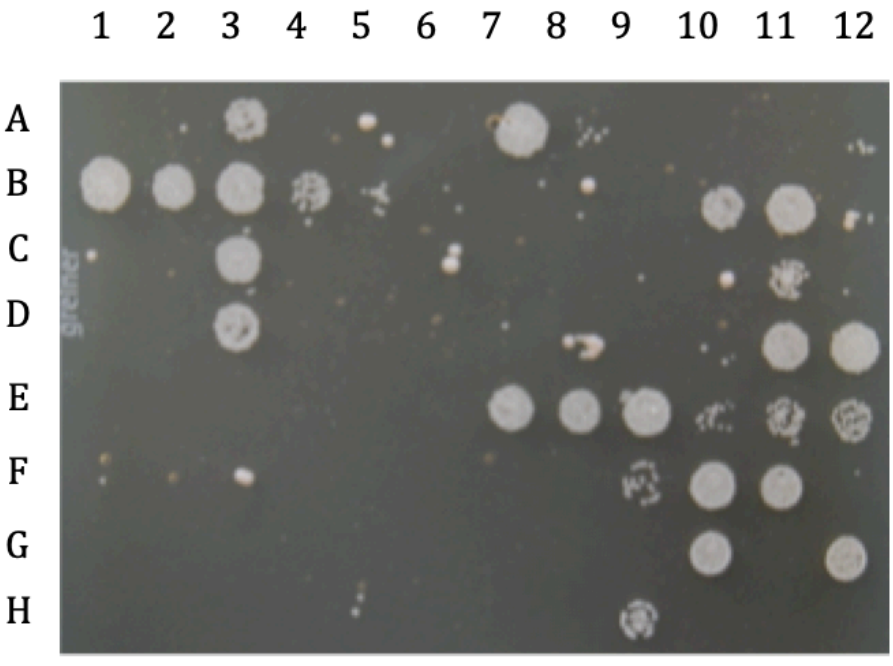


Plate 9

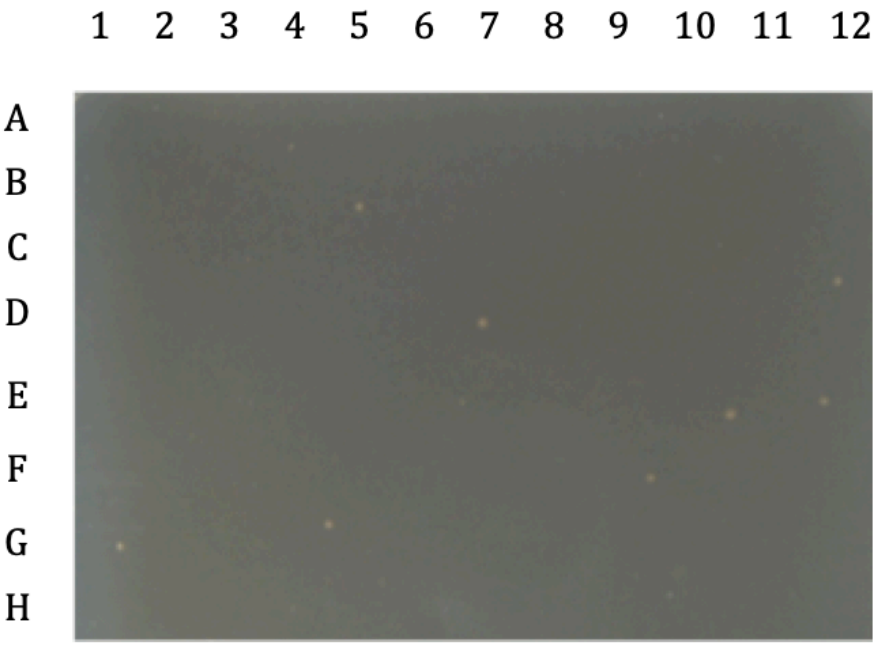


Plate 10

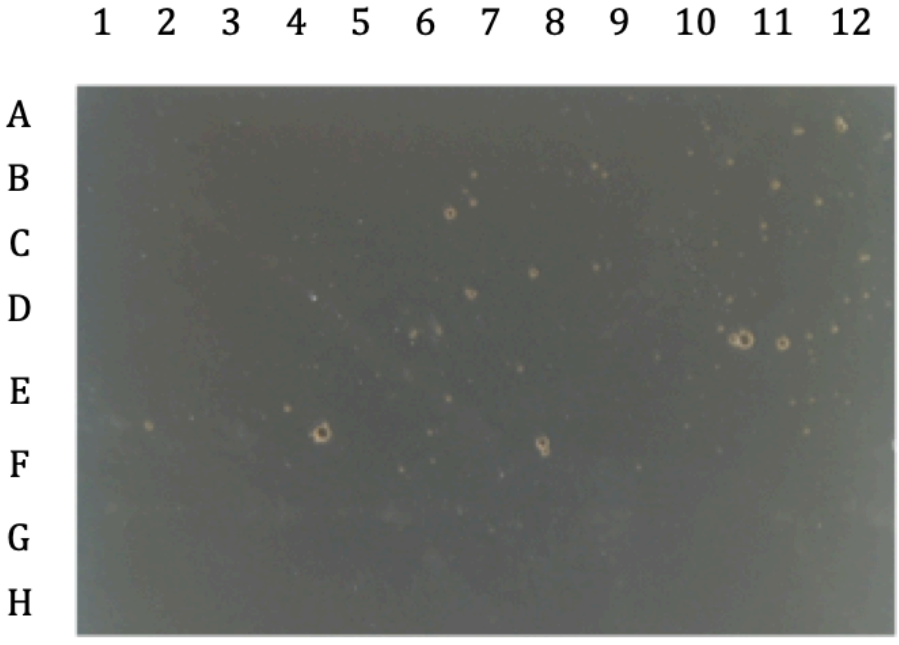


Plate 11

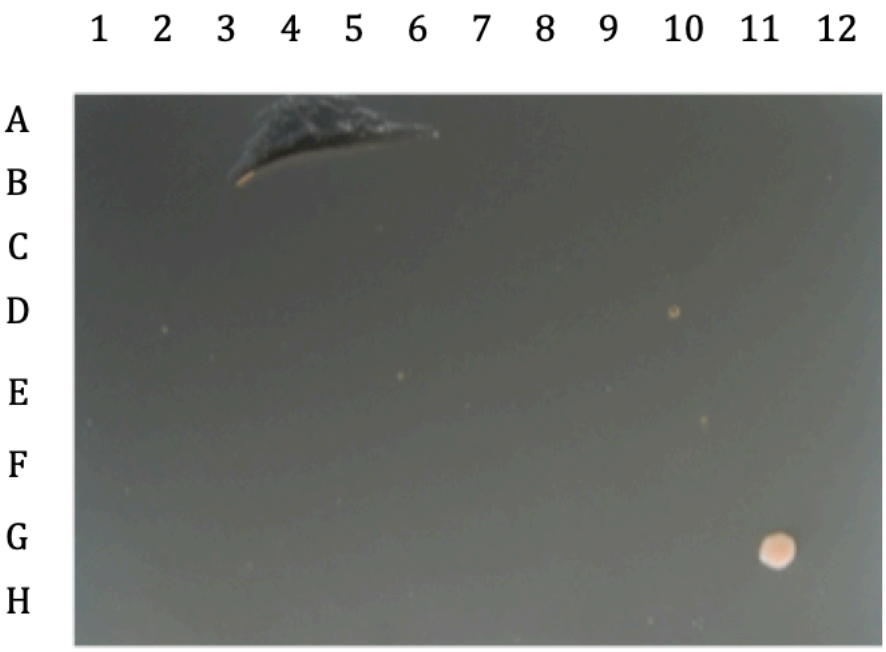


Plate 12

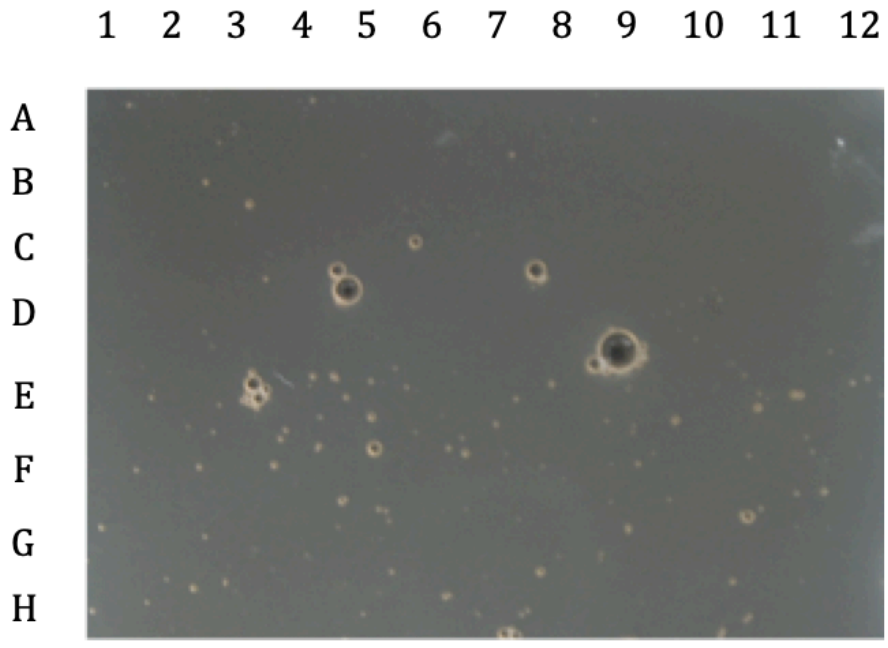


Plate 13

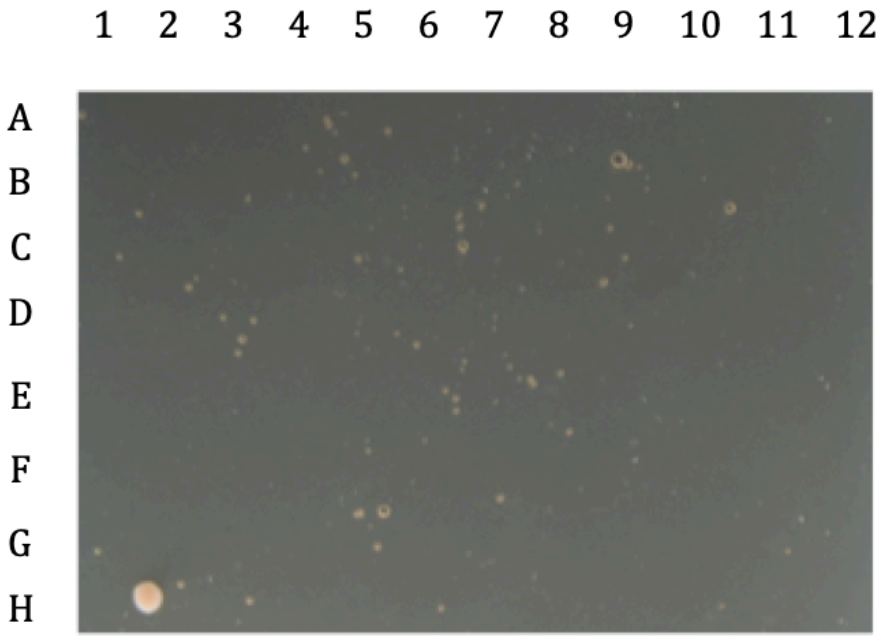
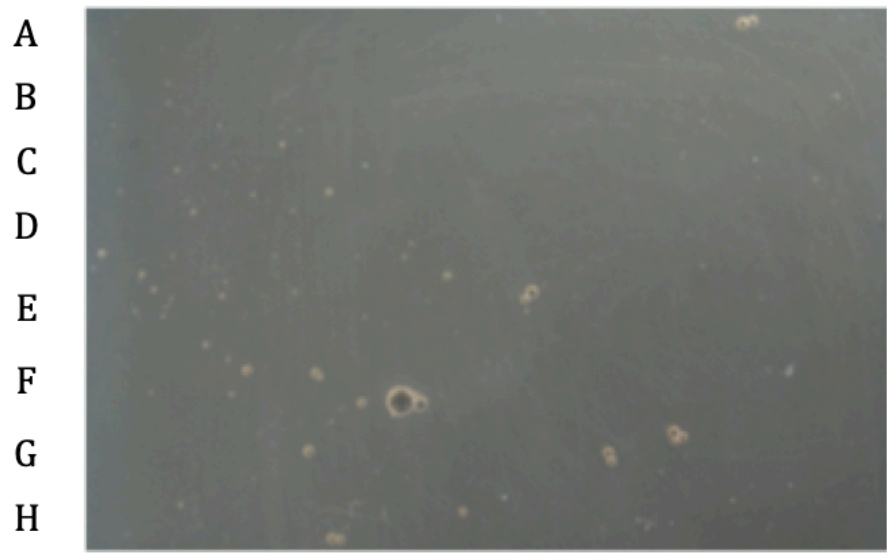
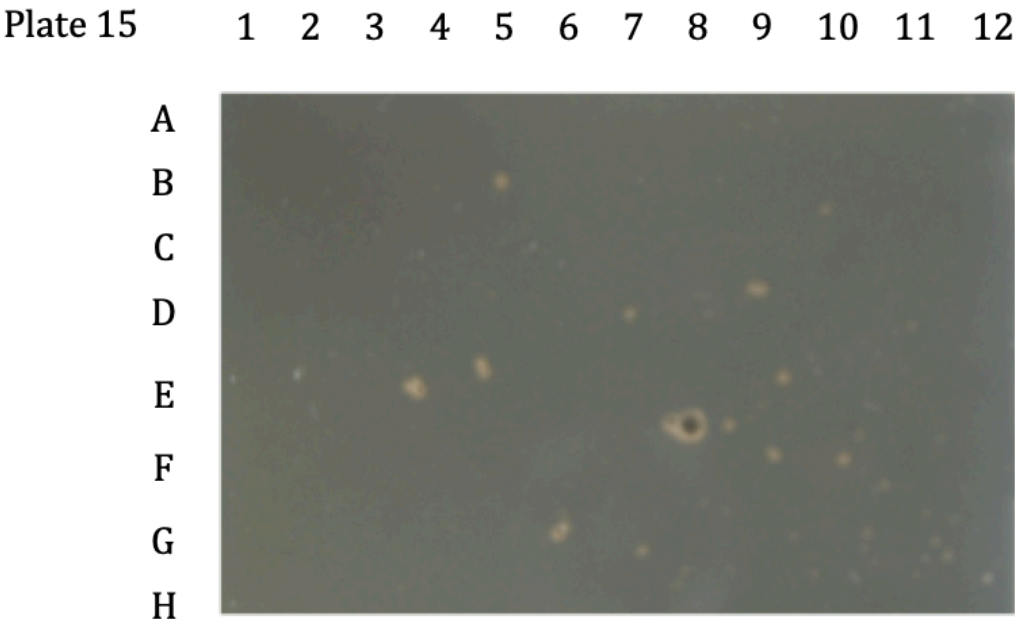


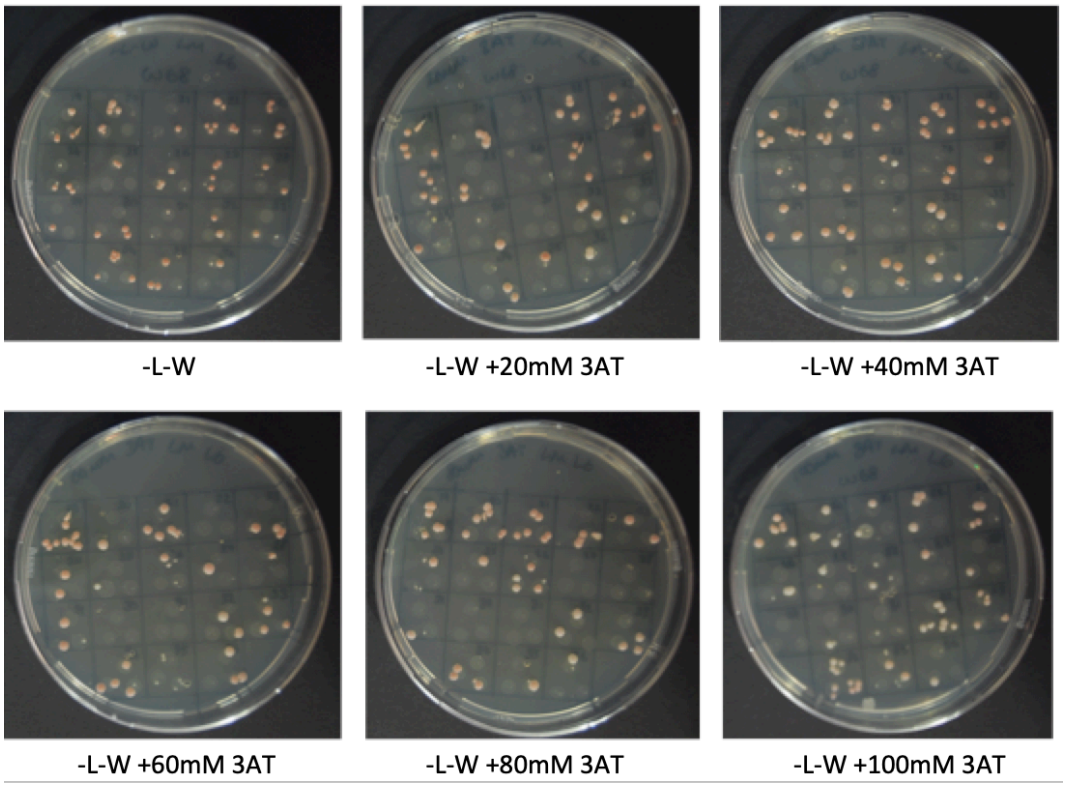
Plate 14

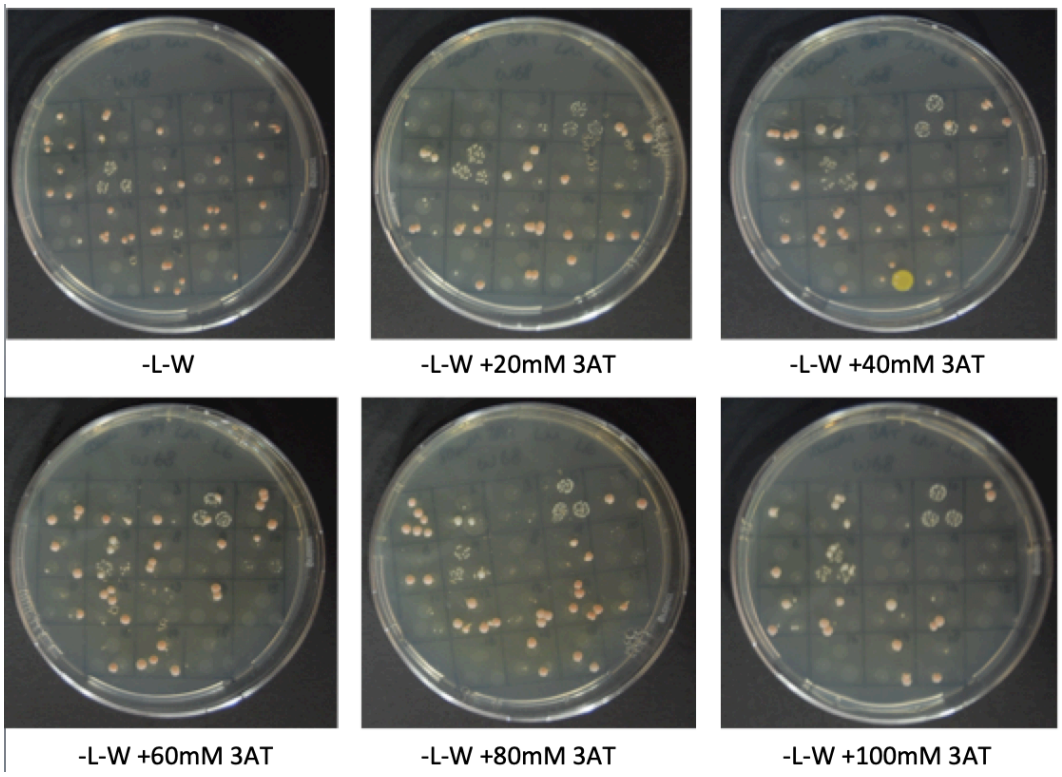


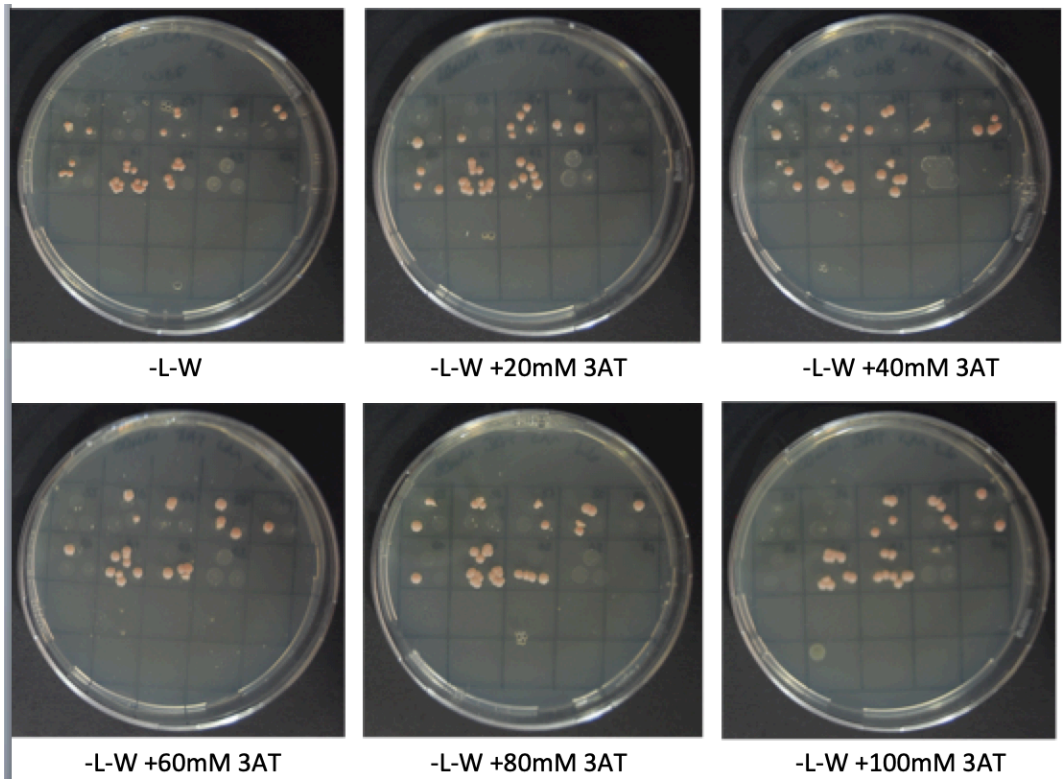


Y1H with TaWRKY9 promoter and a library of 1,200 Arabidopsis thaliana TFs. After mating the yeast was grown in liquid culture (with mating selection) before being stamped onto plates containing selection media (20mM 3AT in –L-W-H minimal SD media). Plates were grown for 4 days at 28^{0}C before photos were taken.

Supplemental figure 8







Pictures of Y1H plates for TaWRKY9 promoter and 68 TFs selected based on preliminary screening. After mating the yeast was grown in liquid culture (with mating selection) before being pipetted (in 5µl) onto plates containing increasing selection pressures (0-100mM 3AT in –L-W-H minimal SD media). Plates were grown for 4 days at 28^{0}C before photos were taken.